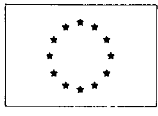

COMMISSION OF THE EUROPEAN COMMUNITIES

EURATOM

RADIATION PROTECTION

PROGRAMME

PROGRESS REPORT

1985-89

Volume 2

EUR 13268 DE/EN/FR

COMISIÓN DE LAS COMUNIDADES EUROPEAS
KOMMISSIONEN FOR DE EUROPÆISKE FÆLLESSKABER
KOMMISSION DER EUROPÄISCHEN GEMEINSCHAFTEN
ΕΠΙΤΡΟΠΗ ΤΩΝ ΕΥΡΩΠΑΪΚΩΝ ΚΟΙΝΟΤΗΤΩΝ
COMMISSION OF THE EUROPEAN COMMUNITIES
COMMISSION DES COMMUNAUTÉS EUROPÉENNES
COMMISSIONE DELLE COMUNITÀ EUROPEE
COMMISSIE VAN DE EUROPESE GEMEENSCHAPPEN
COMISSÃO DAS COMUNIDADES EUROPEIAS

EURATOM

Relación de actividades
Programa

PROTECCIÓN RADIOLÓGICA

Beretning
Program

STRÅLINGSBESKYTTELSE

Tätigkeitsbericht
Programm

STRAHLENSCHUTZ

Έκθεση πεπραγμένων
Πρόγραμμα

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Rapport d'activité
Programme

RADIOPROTECTION

Rapporto d'attività
Programma

RADIOPROTEZIONE

Verslag van de werkzaamheden
Programma

STRALINGSBESCHERMING

Relatório de actividades
Programa

RADIOPROTECÇÃO

1985-89

Volume 2

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NON-STOCHASTIC EFFECTS OF IONIZING RADIATION

EFFETS NON-STOCHASTIQUES DES RAYONNEMENTS IONISANTS

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-C-187-B

Univ. Catholique Louvain-la-Neuve
Halles Universitaires
Place de l'Université 1
B-1348 Louvain-la-Neuve

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30 Clos Chapelle aux Champs
B-1200 Bruxelles

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Title of the research contract:

Consequences of irradiation on the immune system. Prevention and treatment of its effect.

List of projects:

1. Role of B lymphocytes in chronic radiation damage.

Title of the project no.:

ROLE OF B LYMPHOCYTES IN CHRONIC RADIATION DAMAGE.

Head(s) of project:

Prof. H. BAZIN

Scientific staff:

Dr P. MANOUVRIEZ
B. VANHOVE
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I. Objectives of the project:

Injuries of the immune system are a well known consequence of exposure to ionizing radiations. As an immune response is the result of a series of events which imply a number of cell populations, it is necessary to identify them and to analyse their respective radiosensitivities. The present program is focused on the humoral responses and especially on the B lymphocytes which can be divided in various subpopulations, depending on their membrane receptors, mobilities, localizations in the peripheral lymphoid organs, and evidently their respective physiological roles. The main objective of this program is to restore the normal function of the system in order to protect immunosuppressed patients against infections.

II. Objectives for the reporting period:

The reporting period was devoted to study:

- B lymphocytes of rats after a chronic exposure of gamma rays given at a dose rate of 12, 20 and 30 cGy per day.

- B lymphocytes of rats after a single exposure to a sublethal dose of irradiation given at a high dose rate.

- Restoration of B lymphocyte cell populations in irradiated rats after intravenous injection of B lymphocytes from different origins.

- Long term effects of prenatal or early postnatal irradiation on the immune system of rats.

- Long term effects of prenatal or early postnatal irradiation on the circulating T lymphocytes of rats.

III. Progress achieved:

3.1. Chronic exposure of gamma rays given at a low dose rate

Irradiations to cobalt gamma rays were given to Sprague-Dawley male rats at a daily doses of 0, 0.07, 0.12, 0.20 or 0.30 Gy for a period of up to 90 or 135 days. The observed effects increased with the daily dose and the duration of irradiation. However, at a daily dose of 0.07 Gy, as well as a daily dose of 0.12 Gy for 30 days, no clear effect was observed, in particular all rats showed visible follicles with germinal centres and well developed marginal zone cell populations. At all other doses and durations, there was a visible decrease of the B lymphocyte cell populations, with a much higher susceptibility of the marginal zone B population than of the follicular zone B population.

3.2. Exposure to a single dose given at a high dose rate

Spleen IgM plasma cells (as seen in Fig.1) did not change in number during the months following 5 Gy whole body irradiation by comparison to the controls. Contrariwise, a large and significant increase of IgA plasma cells was detected in the same organ, reaching its maximum at day 20 after irradiation (Fig.1).

In the mesenteric lymph nodes, the number of IgM plasma cells fluctuated between 10 and 100 cells per section, whatever the time after irradiation was. IgA plasma cells, in the same lymph nodes, were difficult to count. However, their number was constantly high, between 5×10^2 to 10^3 cells per section, whether in irradiated rats or in control rats.

In the intestinal tract, there was a clear reduction in length of the villi associated with a size reduction of the Peyer's patches. An approximately equal number of IgA plasma cells could be seen in the lamina propria of the mucosae from day 1 to day 7 after irradiation. From day 10 to day 15 after irradiation, there was an enormous increase of IgA plasma cells which returned to normal at day 20 as well as the size of the villus. The number of IgA plasma cells in and around the Peyer's patches was always limited and not significantly different from those of the controls.

The serum concentrations of the IgM and IgA are given in Fig.1.
The IgM and IgG serum levels showed a rather similar pattern, both of these immunoglobulin isotypes slightly increased with time after irradiation. However, the control rats showed a similar increase of their IgM and IgG serum levels and so, no significant increase was detected in the irradiated rats by comparison to the control ones.
On the contrary, the IgA serum level of the irradiated rats decreased in the days after irradiation and then, sharply increased up to day 25. This increase was followed by a decrease. Their IgA serum levels returned, after day 30, to the level of the control rats.

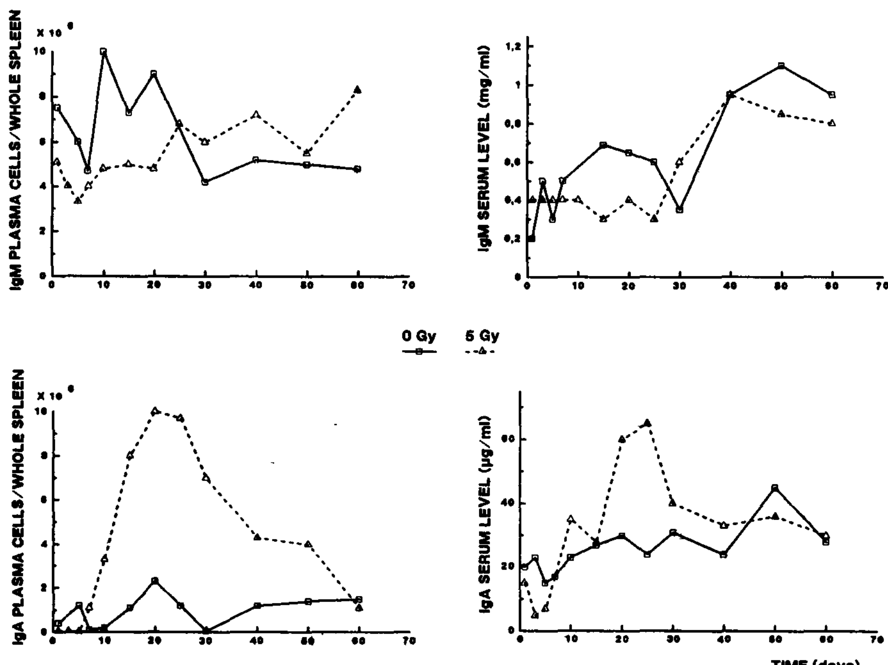


Figure 1:
Number of IgM (top, left) and IgA (bottom, left) plasma cells and IgM (top, right) and IgA (bottom, right) serum levels in irradiated (5 Gy whole body irradiation) and control rats, as a function of time after irradiation.

3.3. Restoration of B cell population in irradiated rats

The effects of lymphocyte transplantation on the restoration of the B lymphoid system after a 5 Gy whole body irradiation have been analysed in rats. Male and female 3 month-old Fisher rats have been irradiated with a gamma ray machine (137 caesium source). The standard radiation dose delivered was 5 Gy.
In the first experiment, cells from histocompatible rats, either 3.5 x 10^8 cells from spleen or 0.5 x 10^8 cells from mesenteric lymph node were intravenously inoculated, 4 hours after the irradiation. On days 1,3,7 after irradiation and transplantation, irradiated or non-irradiated rats were sacrified. Serum immunoglobulins were measured and spleen, mesenteric lymph nodes and Peyer's patches were taken for immunohistological studies.
The immunoglobulin serum levels of these animals are given in Fig.2.
Tissue sections from spleen showed a clear appearance of follicular cells already at day 1 after irradiation, but the marginal zone cells appeared at day 3 in the spleen cell transplanted rats and at day 6 in irradiated rats in which mesenteric lymph node cells were inoculated.
In another series of experiments, B lymphocytes have been transplanted in a congenic rat system that we have developed and in which, it is possible

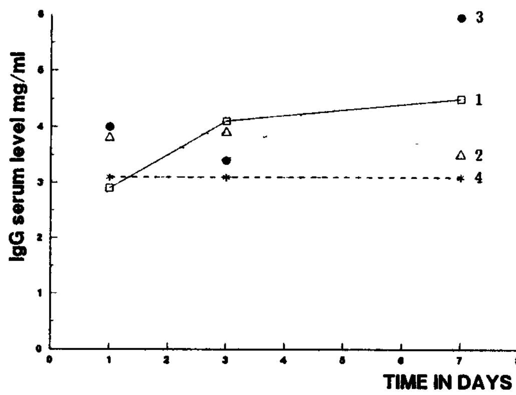
to transfer B lymphoid cells and to follow these cells and their descendants. In rat species, a major immunoglobulin allotypy is located on the constant part of the kappa light chain. Moreover, kappa light chain represents the 95% of the immunoglobulin light chain isotype in this species. Two allotypes have been described, the IgK-1a allotype being carried by the LOU strain and the IgK-1b allotype by, among others, the OKA strain (Beckers et al. Immunochemistry 1974,11,605). We have developed a congenic strain to the LOU/C rats having the kappa light chain of the OKA strain. Both strains, LOU/C with the IgK-1a locus and LOU/C.IgK-1b(OKA), are perfectly histocompatible as judged by reciprocal skin graft. We have also developed hybridomas synthetizing monoclonal antibodies which can react only with immunoglobulin molecule carrying the kappa light chain either of the IgK-1a allotype or the IgK-1b allotype. By transplanting lymphoid cells from rats of one strain in the other strain, it is possible to follow these cells and their secreted immunoglobulins but also their descendant cells and their products of secretion.

Lymphoid cells of the LOU/C(IgK-1a) rats have been inoculated in LOU/C.IgK-1b(OKA) rats and the results given in table 1.

Table 1: Lymphocytes transplanted from LOU/C (IgK-1a) rats into irradiated LOU/C.IgK-1b(OKA) rats, as seen by the MARK-3 (mouse anti-rat kappa light chain of the IgK-1b allotype number 3).

Time after transplantation (in days)	1	3	5	7	10	15	20	25	30	40	50	60
Follicular zone*	++	++	++	+++	+++	+++	++++	++++	++++	++++	++++	++++
Marginal zone*	0	+	+	++	+++	+++	++++	++++	++++	++++	++++	++++
IgM plasma cells°	0	0	0	++	++	++	+++	+++	+++	+++	+++	+++

*The number of cells per tissue section is given in arbitrary unit by comparison to the control non irradiated rats.
°The number of cells is given per tissue section: 0: no cells; ++: 25-50 cells; +++: >50.



<u>Figure 2</u>: IgG serum level of control (1) or irradiated (2) restored with spleen (3) or mesenteric lymph node cells (4) in function of time after irradiation.

In conclusion, the follicular cell compartment (circulating B cells, mu positive and delta positive) can be restored very quickly by spleen as well as lymph node cells. The marginal zone cells do not reach directly their normal localization but need two or three days before being localized in their normal environment. When animals are restored with lymph node cells, they do not recover marginal zone cells before day 6 after irradiation. We hope to understand the signification of this period of time.

3.4. Long term effects of prenatal or early post natal irradiation

In collaboration with F. Vander Plaetse and R. Hooghe (Pathology Section, Biology Dept, Study Center for Nuclear Energy, B-2400 Mol, Belgium), we have looked for long term sequelae in the immune system of rat that had been irradiated on a single occasion (0-2 Gy, whole body irradiation, X-rays) during prenatal or early postnatal life. At the age of 2 months, the histology of the spleen was normal, and so were the amounts and distribution of B and T lymphocytes. The serum immunoglobulin levels were not altered, even when the different isotypes were considered. At the age of 10 weeks, rats were immunized with a T-dependent or a T-independent dinitrophenylated-carrier antigen. Normal levels of specific antibodies were generated in all groups of animals. Thus no long term immunodeficiency could be documented in rats that had received up to 2 Gy before or early after birth.

3.5. Long term effects of prenatal or early postnatal irradiation on circulating T lymphocyte subpopulations

We have looked for long term sequelae in the immune system of rats that had been X-irradiated (0-2 Gy whole body irradiation) once or twice during prenatal or early postnatal life, in collaboration with R. Hooghe. At an age of 15 months, blood was taken for routine haematological analysis. In addition, T-cell subpopulations were analyzed at the FACScan with monoclonal antibodies W3-25, MRC OX-8 and MRC OX-19, used respectively as CD4, CD8 and pan-T reagents. In control males, 29% of blood lymphocytes expressed CD4, 33% CD8 and 39 the pan-T marker. In females, the corresponding figures were 39, 34 and 53. These important differences between males and females were not affected by most irradiation protocols. However, females given 1 Gy 6 days after birth had only 31% CD4, 26% CD8 and 39% lymphocytes expressing the pan-T marker. In contrast, females irradiated twice (1 or 2 Gy, 16 or 20 days after conception and 6 days after birth) often had a clear-cut increase of the CD8 positive population (46%) and a decrease of the CD4 population (25-32%) and of the OX-19 (pan-T) population (29-35%). In conclusion, a single irradiation (1 or 2 Gy) during prenatal or early postnatal life had little effect, except for a decrease of all T-cell populations in females irradiated 6 days after birth. Females irradiated twice showed an increase in CD8 and a decrease in CD4 and "pan-T" populations. The fact that no effect was seen in males suggests a possible role for sex hormones in long term haematological effects of irradiation.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-C-057-UK

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Telephone number: 01-251 1184

Title of the research contract:

Stochastic and non-stochastic effects of alpha and beta radiation on mouse skin.

List of projects:

1. Non-stochastic effects of alpha and beta radiation on mouse skin.
2. Stochastic effects of alpha and beta radiation on mouse skin.

EURATOM FINAL REPORT.

NON-STOCHASTIC EFFECTS OF ALPHA AND BETA RADIATION ON MOUSE SKIN.

Introduction.

The problems arising in the nuclear power and related industries, from skin contamination with radioactive particles, continue to be a cause for concern. The acute effects (erythema, oedema, pigmentation, desquamation and ulceration) following penetrating irradiation from large uniform sources have been well documented, and the severity and duration of the reactions and the dose response relationships are known to be dependent on dose rate, dose protraction and the volume of tissue irradiated (Jammet et al, 1986). However, much less is known of the effects of less penetrating alpha and beta sources, although they play a major role in accidental exposures e.g. Goiania and Chernobyl, as well as in some occupational environments (UNSCEAR, 1988; Charles, 1986).

The guidance from 1977-1984, offered by ICRP for skin exposure, has been widely criticised, especially its lack of clarity over dose averaging following exposure of areas of <<1cm^2 of skin (Wells, 1986). There are very few human or animal data on the effect of varying the area of skin irradiated for different alpha and beta energies and the purpose of this study was to fill this experimental gap. The work has been collaborative with Drs.M.W.Charles and J.Wells of the CEGB Berkeley Nuclear Laboratories and with Dr.J. Hopewell of the CRC Laboratory at Oxford, the latter having studied early and late non-stochastic effects in pig skin.

The precise aim of the work has been to attempt to provide quantitative answers to the following practical questions: Is there a minimum area that needs to be irradiated to induce moist desquamation?; What are the threshold doses for this effect, and how do these vary with the energy of the radiation and the area of skin exposed? To do this, we have generated dose response curves in mouse skin for three very

different energy beta emitters (promethium-147, thulium-170 and strontium/yttrium-90) and for curium-244 alpha particles.

Materials and Methodology.

Experimental animals.

Male, random-bred SAS/4 albino mice, 11 to 12 weeks old, were used in all experiments. They were obtained from a colony at the Medical College of St.Bartholomew's Hospital. Prior to irradiation, an area of dorsolateral skin was depilated with a depilatory agent, Veet (Reckitt Toiletries Ltd., Hull, U.K.). This procedure triggers resting (telogen) follicles into the active (anagen) cycle, thus ensuring that the epidermis and dermis were of a known thickness and that the hair follicles were at a known depth in the skin. The mice were irradiated immediately after depilation while the follicles were still in telogen. The average epidermal thickness at this time is 10.7 ± 1.6um (SE), dermal thickness is 441 ± 30um and the bases of the hair follicles are at 141 ± 15um (Hansen et al, 1984). These thicknesses take into account the natural elasticity of skin. Any mice showing hair regrowth before day 9 after depilation/irradiation were excluded from the experiments since such early regrowth implied that the follicles had been in anagen and not telogen at the time of irradiation.

For each source and dose point, 12-18 mice were used and they were observed daily for 6 weeks for acute skin reaction with the unirradiated flank acting as control skin.

Radioactive sources.

The beta sources used were strontium-90/yttrium-90, thulium-170 and promethium-147 (E_{max} = 2.27, 0.97 and 0.23MeV respectively). The strontium sources were produced by Amersham International plc and were circular, with diameters between 1 and 22.5mm. The thulium-170 sources were produced by Drs.M.W. Charles and J.Wells of the CEGB, by the neutron activation of pure thulium-169 in the form of a 0.1mm thick foil. The circular Tm-170 sources had diameters between 0.1 and 9mm and were mounted on pure aluminium rods. The 15mm circular promethium source was also produced by Amersham International plc. To enable the diameter of the source to be varied,

shields of aluminium and melinex (120:120um), with either 2, 5 or 9mm diameter circular holes punched in each, were fixed across the source, with the melinex in direct source contact to reduce scatter.

The curium-244 alpha source (E_{max} = 5.8MeV) was made by UKAEA by neutron activation and was a large 2 x 4cm planar source.

The dosimetry of all sources was carried out using an extrapolation ionisation chamber using a range of collector electrodes, from 1 to 10mm, and all beta dose measurements were central axis, made with a 16um tissue-equivalent window, and so the doses quoted in this paper actually refer to the doses at this depth in the skin.

The dosimetry of the alpha sources was calculated at 260Gy hr^{-1} ($\pm$10%) at the skin surface, 179Gy hr^{-1} at 10um and zero at 30um into the skin.

Results.

Figs.1, 2 and 3 show the dose response curves for the moist desquamative skin reaction following strontium, thulium and promethium exposures respectively. All of the dose response curves show a marked area effect, i.e. as the area irradiated increases, the dose required to produce moist desquamation decreases. Table I gives the approximate threshold doses for each of the sources and the doses required to produce moist desquamation in 50% of the mice (MD-50's).

A critical analysis of the temporal sequence of the acute responses for promethium, thulium and strontium was carried out using a comparison of three diameters (2mm, 5mm and 9 or 11mm) for each of the sources. Each of the thulium and strontium sources demonstrated three distinct, dose dependent phases of response, and using the 11mm strontium data as examples, the details of the three phases are briefly described:

1. At the lowest doses ($\leq$25Gy), no moist desquamation was seen and the mice exhibited only a low level dry desquamative reaction.

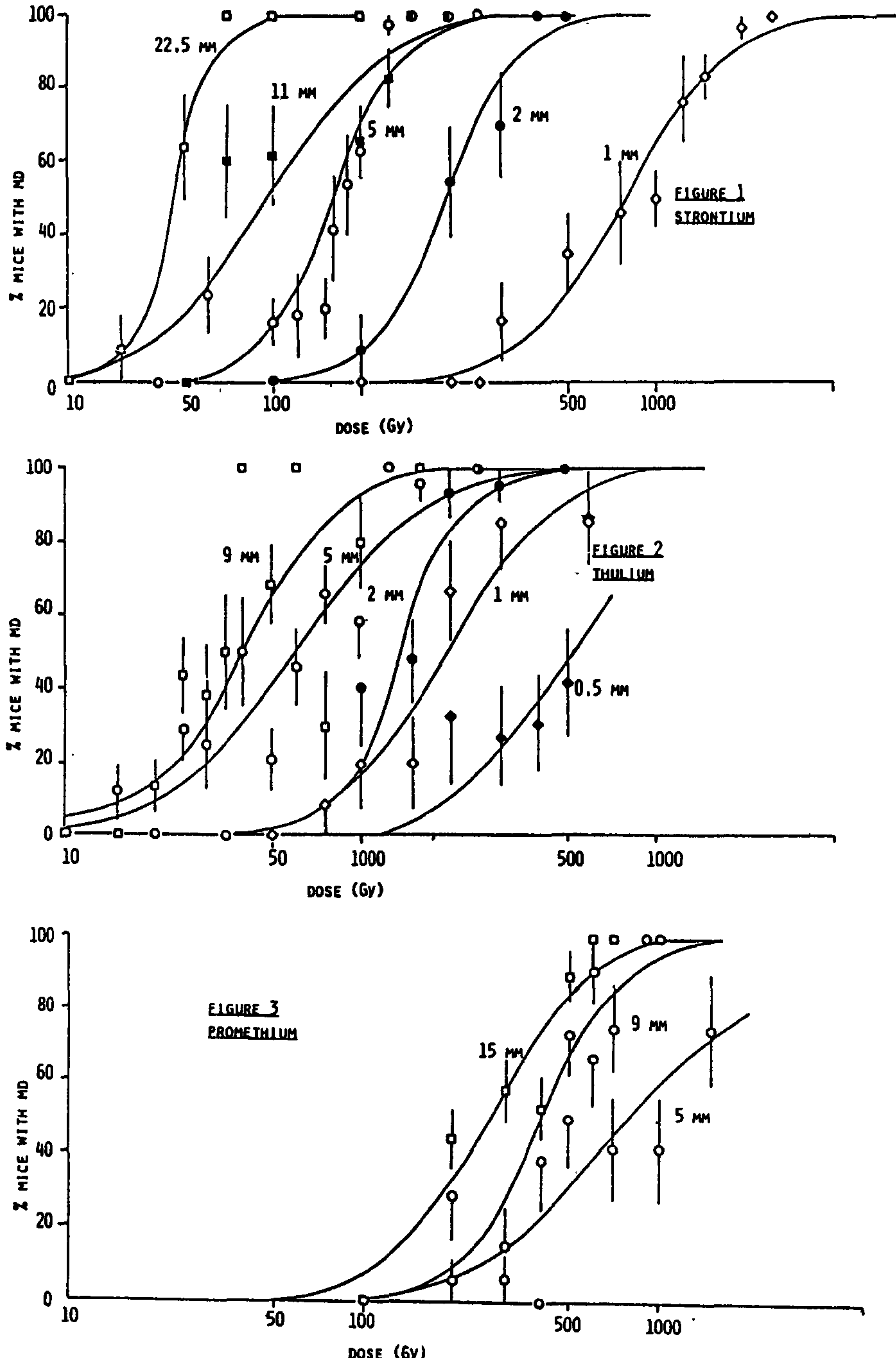
% MICE WITH MD
DOSE (Gy)
22.5 MM
11 MM
5 MM
2 MM
1 MM
FIGURE 1
STRONTIUM
9 MM
5 MM
2 MM
1 MM
0.5 MM
FIGURE 2
THULIUM
FIGURE 3
PROMETHIUM
15 MM
9 MM
5 MM

Table I. Threshold and MD-50 Doses for Acute Skin Reactions.

Source	Threshold Dose (Gy)	MD-50 Dose (Gy)
1mm Sr-90	140	710
2mm Sr-90	90	250
5mm Sr-90	30	90
11mm Sr-90	20	48
22.5mm Sr-90	10	25
0.1mm Tm-170	120	-
0.5mm Tm-170	75	610
1mm Tm-170	60	230
2mm Tm-170	45	130
5mm Tm-170	15	60
9mm Tm-170	10	30
2mm Pm-147	-	-
5mm Pm-147	170	800
9mm Pm-147	140	400
15mm Pm-147	100	280

2. At intermediate doses (35-100Gy), there was little variation in the degree of moist desquamation, with some two third's of the mice showing a synchronised, short duration, moist desquamative response. The onset of this reaction at 12 days and its duration of 8-9 days was independent of dose level in this range.

3. At the highest doses (≥125Gy), 100% of the mice showed moist desquamation, its onset being as early as 6 to 8 days post-irradiation and the duration of the reaction was 6 weeks (@ 125Gy), and even longer for the higher doses. These persistant moist desquamative reactions were associated in many cases with an underlying ulceration.

This sequence of dose dependent skin reactions was also observed with the other strontium sources, but as the area irradiated was reduced, the doses to produce phases 1, 2 and 3 increased.

The results for the less penetrating betas from thulium produced the same three responses at dose levels similar to those for strontium, although the level of response in the intermediate phase was lower, i.e. following thulium irradiation, only 30% of the mice showed the synchronised, short duration moist desquamative response, in comparison with 60% of the mice following strontium exposure.

Finally, with promethium, the least penetrating beta emitter, moist desquamation was not inducible at doses below 100Gy and although the larger sources (see fig.3) produced an area- and dose dependent increase in reaction levels up to a 100% response, there was no transition to the severe phase 3, the prolonged desquamative/ulcerative reaction.

The acute reaction studies for the 8cm² curium-244 source showed that even at the highest dose level used (180Gy), none of the animals exhibited moist desquamation. The acute response began as a mild erythema on day 2 post-irradiation, and reached a peak response on day 6, when some 66% of the mice exhibited a number of dry flakes. Flaking was observed until approximately day 10, thereafter only mild erythema was seen persisting in only a few mice for two or three days. At lower dose levels (120 and 80Gy), there was a dose related increase in the time to reaction onset and a dose related reduction in the reaction duration, although the peak in response still appeared on days 6 and 7 respectively.

At dose levels below 80Gy, the only discernible gross macroscopic reaction was a small degree of "mottling" in the skin coloration. At all dose levels, the acute reaction became difficult to score beyond 12 days post-irradiation since there was no disruption to the hair growth cycle beyond a slight delay, so that at this time the hair regrowth obscured the irradiated field.

This inability of curium alphas to elicit moist desquamation, even using large sources, meant that it was unnecessary to investigate smaller area alpha sources. The relative ineffectiveness of the alphas is presumably related to the lack of penetration of the particles - their maximum range being only ~30um into the skin. Such a dose distribution

would leave >95% of the follicle basal cells unirradiated and available for rapid re-epithelialisation of the damaged superficial basal layer.

Discussion.

The data in this paper are comparable with some earlier published studies (Coggle et al, 1984) and confirm that strontium and thulium sources greater than 1mm in diameter are equally efficient at producing acute moist desquamation despite the greater penetration of the strontium betas. In contrast, the much less energetic betas of promethium-147 required much higher doses to produce the same levels of acute skin reaction. But once again there was a pronounced area effect and with the smallest source (2mm diameter), it was impossible to produce exudative epidermatitis in the mouse skin, even at doses as high as 1500Gy. A similar situation was found with the two smaller thulium-170 sources - 0.1 and 0.5mm in diameter - where the threshold dose, as fitted by eye for the 0.1mm source, is approximately 120Gy, whilst the MD-50 value for the 0.5mm source is 610Gy, with a threshold of 75Gy. As was the case for the smaller promethium-147 sources (≤5mm), the highest doses did not produce 100% moist desquamation, although the low dose rates of the small thulium sources prevented further investigation at higher doses.

From Table I, it can be seen that the 1mm Sr-90 MD-50 value of 710Gy is much higher than the 1mm Tm-170 MD-50 value of 230Gy. This 'anomalous' result, with the more penetrating betas of the strontium source being less effective than those of the thulium, has still to be resolved, either physically, with a reassessment of the dosimetry of the sources or biologically, using histology to check any degree of under-scoring of the small lesions that might account for the result.

To explain these area and energy effects, we propose that moist desquamative lesions heal principally by two processes: (a) by cell migration and division from basal cells at the periphery of the irradiated field;

and/or (b) by cell migration and division from undamaged basal cells from within the field, from the epidermis and hair follicles.

Cell migration from the periphery of the area has been shown by a number of authors, e.g. Franklin and Coultas (1984) and occurs in mice, pigs and guinea-pigs at rates of between 0.1 and 0.4mm day^{-1} (Coggle et al, 1984; Devik, 1977; Osanov et al, 1976). Migration and division of cells within the field has similarly been demonstrated by other authors, e.g. Al-Barwari and Potten using their micro-colony assay (1976).

The working hypothesis also links the two repair mechanisms mentioned above to the energy of the radionuclides. No area effect is seen where the dose or energy of the beta particles is insufficient to damage the clonogenic basal cells, be they epidermal or follicular. As the energy of the electrons increases, gaps start to appear within the irradiated field; repair now occurs either from pockets of basal cells within the field or from the periphery and the relative roles of each mode of repair are dependent on both the energy of the radionuclide and the area of the field. The greatest area effect will be seen at the higher energies until, ultimately, repair is totally dependent on peripheral proliferation, when we will see the maximum area effect. This effect is also illustrated in the skin clonogen work of Withers (1967) who isolated areas of skin by lethally irradiating an annulus around the field of study, so preventing cell migration.

Our work has also produced a number of results that point to the involvement of dermal damage as contributing to the effects especially at the higher dose level, 'phase 3' reactions referred to previously. The depth dose distributions of both thulium and strontium do not stop at the basal layer and it seems plausible to suggest that the restorative capacity of the basal cells depends on the integrity of the dermal elements and that this is particularly important in producing the more persistant severe desquamative/ulcerative lesions at the higher doses and larger areas. Conversely, the inability of the weaker, less penetrating promethium betas to

elicit significant dermal damage may explain why severe phase 3 damage was not inducible even with the highest doses and largest sources. Also, the lack of effect is pronounced due to the presence of surviving epithelial cells always available in the irradiated area from the hair follicles.

From the mouse studies and the non-stochastic pig skin studies of our Oxford collaborates, it would not seem possible to choose a single area and depth dose over which to average dose which would then provide a single predictive indicator of skin damage for all source sizes and energies (see Charles et al, 1989). This is presumably due to the variety and complexity of the biological endpoints of the acute skin reactions. For the large area sources, the threshold doses of ~ 10-20Gy (see Table I) are in the region of the current life time ICRP skin dose limit of 20Gy. For chronic, occupational exposures, the sparing due to repair is expected to increase the thresholds somewhat. For small sources, weak radiations and where the exact skin area is unlikely to be persistently exposed, in practice the threshold doses are going to be orders of magnitude in excess of the current annual ICRP dose limits of 0.5Gy. These conclusions should be reassuring to those in the nuclear and other radiological industries who are currently concerned over potential health effects in skin of particulate irradiation.

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Publications:

Recent Trends in Radiobiology of Skin and Repurcussions for Dose Limitation and Personal Dosimetry. M.W. Charles, J.W. Hopewell, J. Wells and J.E. Coggle in 'Radiation Protection in Theory and Practice' (IOP Publishing Ltd) 419-424, (1989).

EURATOM FINAL REPORT.

STOCHASTIC EFFECTS OF ALPHA AND BETA RADIATION ON MOUSE SKIN.

Introduction.

Cancer induction is the most significant risk following irradiation in man. Human exposure to radiation is rarely uniform over the whole body or even over a whole tissue so that the epidemiological data used in cancer risk analysis are usually derived from non-uniform or partial body exposure. Consequently, it is important, for radiation protection purposes, to design experiments involving comparisons of uniform and non-uniform radiation exposure. This is especially relevant in assessing tumour induction in the skin, where possible sources of localised skin exposure include small radiotherapy sources, radiopharmaceuticals, collimated microbeams and both fission and activated products from nuclear reactors, neutron generators and associated facilities. For radioactive particulates emitting radiation with a very limited range, it is possible that high doses may be received by very small volumes of skin, while most of the skin will receive no dose. It has been suggested that such extremely inhomogeneous radiation would not have the same effect as a dose averaged over the whole skin surface.

The carcinogenic effect of "hot particles" is not a new problem; it was considered by the International Commission on Radiological Protection in 1969 (ICRP, 1969) and has been a controversial issue ever since (Coggle et al, 1984). However, non-uniform irradiation of the skin is a hazard, commonly encountered in radiation protection, which has received very little attention (Wells, 1986), probably because of the current ICRP view that skin cancer is a negligible risk in comparison with such non-stochastic effects as ulceration and dermatitis. For skin exposed over areas of less than $1cm^2$, the current lack of guidance in ICRP (1977) reflects the lack of available information on non-uniform exposure.

This lack of specific guidelines has meant that the nuclear industry and other authorities have based their

operational procedures and permitted levels of skin exposure on the assumption that the risk from radioactive particles is not affected by the spatial distribution of the resultant radiation. This view is, however, not universally accepted and has given rise to considerable debate (Wells, 1986).

To investigate the problems posed by highly localised skin exposure, a collaborative project was set up between the Central Electricity Generating Board's Berkeley Nuclear Laboratories, the Radiation Biology Department at the Medical College of St.Bartholomew's Hospital and the CRC Normal Tissue Radiobiology Research Group at Oxford. The results of the study of non-stochastic effects in pig and mouse have recently been reported elsewhere (Hopewell, 1990; Williams et al, 1990).

This paper presents data on the stochastic effects of thulium-170 beta irradiation on the skin of several different strains of mouse. The stochastic effects of alpha particles from a curium-244 source on a single mouse strain are also presented. Dose response curves are given for skin tumour induction following uniform and non-uniform modes of exposure.

Materials and Methodology.

Experimental animals.

In most experiments, male random bred SAS/4 albino mice, 10-12 weeks old, were used; male CD1, male and female C57Bl/6 and male CBA/Ca strains were used in the comparative study.

Radiation sources.

In the study to compare uniform with non-uniform irradiations, thulium-170 (E_{max}=0.97MeV) was used. The uniform rectangular 3.9 x 2.2cm sources were produced by wrapping 100um thick thulium-169 metal foil in three layers of 15um thick aluminium. Ultrapure materials were used to avoid activation of trace elements. Non-uniform sources were produced as arrays of 8 or 32, 2mm diameter thulium foil discs mounted on an ultrapure 100um thick aluminium sheet of area 4 x 2cm. Source alignment was checked by x-radiography. Following neutron activation, the thulium-170 sources were allowed to cool to allow the ^{28}Al activation product to decay.

Depth doses and dose distributions of the beta sources were measured using an extrapolation ionisation chamber with collecting electrodes of diameters 1mm up to 1cm (minimum area of accuracy is 1.1mm^2) and with ultrathin (30um LiF/ Teflon) thermoluminescent dosimeters. Since the ionisation chamber has a 16um tissue-equivalent window, the measured "surface doses" actually refer to doses at this depth, whilst the beta doses at the base of the dermis are 50% of the skin surface doses.

Using a 1mm electrode, the minimum surface dose rate between individual sources in the 32-source array was measured at 40% of the peak dose and 90% of the mean dose over the whole array. In the case of the 8-source array, each source was independent; peak doses measured with the 1mm electrode were an order of magnitude greater than the mean dose over the whole area. The initial dose rates were 17.3Gy min^{-1} for the uniform source, 18.1 and 4.9Gy min^{-1} for the 32- and 8-source non-uniform arrays respectively. Following calibration, the sources were mounted in lead and aluminium irradiation jigs with interlock and failsafe chambers.

For the alpha particle irradiations, similar 8cm^2 rectangular curium-244 (100uCi) sources were made and covered with a 4um melinex film. The skin surface dose rate was calculated at 260Gy hr^{-1} ($\pm$10%), 179Gy hr^{-1} at 10um and the dose at 30um was zero. These sources were mounted in identical failsafe mouse irradiation jigs.

Irradiation procedure.

Mice were anaesthetised using 62.5mg kg^{-1} body weight intraperitoneal sodium pentobarbitone and an area 5 x 3cm of the right or left flank was depilated using Veet (Reckitt Toiletries Ltd., Hull). The mice were secured to an aluminium tray and placed in the jigs. Control mice were sham irradiated. In the first thulium-170 experiment, involving 1200 SAS/4 mice in groups of 100, doses between 20 and 1000Gy (uniform), 20 to 500Gy (32-array) and 20 to 450Gy (8-array) were given. In the second thulium-170 SAS/4 experiment, doses of 2, 5, 10 and 20Gy were used. In the study of strain differences, groups of 55-65 albino (SAS/4, CD1), brown (CBA/Ca) and black (C57Bl/6) mice were given uniform thulium-

170 beta doses between 12.5 and 100Gy. Finally, SAS/4 mice were used in the curium-244 alpha carcinogenesis experiment where eight dose groups of 75-100 mice were given uniform doses between 2 and 180Gy.

Mice were observed daily for 6 weeks through the acute skin reaction period and then weekly throughout their lifespan for signs of morbidity and for the appearance of a skin tumour in or adjacent to the irradiated area. Post-mortem examination was performed on all animals and all tumours were kept for histopathological classification.

Results.

I. Uniform versus non-uniform thulium-170 data.

None of the 152 control mice developed skin tumours on their irradiated flank skin. However, a total of 389 skin tumours developed in the irradiated mice and Table I shows the spectrum of pathologies induced by the uniform and non-uniform thulium-170 sources.

Fig.1 shows the cumulative skin tumour incidence in the first experimental series at 120-129 weeks post-irradiation as a function of mean skin surface (16um) dose for the uniform, 32- and 8-arrays of thulium-170. These data show that there is no significant reduction in tumour incidence above 300Gy, and so the whole range of uniform data cannot be fitted to any simple single radiobiologically acceptable model.

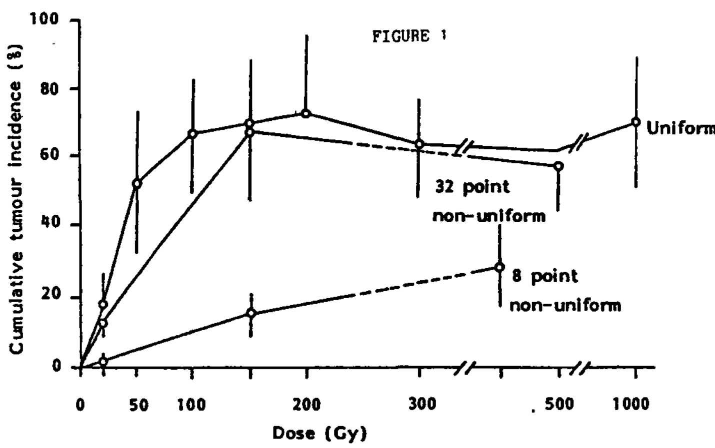


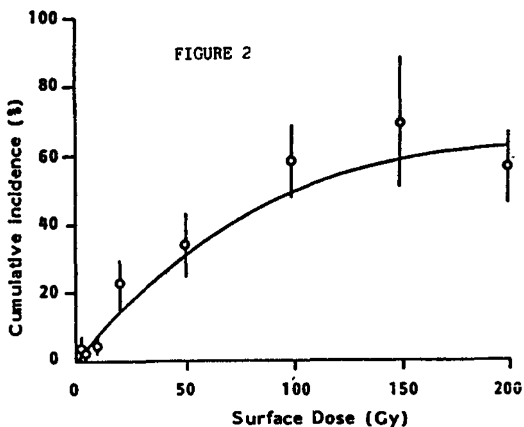
Table I. Pathology of Skin Tumours Induced by Thulium-170 Beta Particles (expressed as %'s).

Tumour type	Uniform source (n=286)	Non-uniform (32-array) (n=75)	Non-uniform (8-array) (n=28)
Squamous cell carcinoma	1.7	4.0	7.1
Squamous papilloma	2.8	1.3	-
Basal cell carcinoma	1.0	-	-
Sub-Total	5.6%	5.3%	7.1%
Rhabdomyosarcoma	62.6	52.0	42.9
Leiomyosarcoma	5.6	5.3	7.1
Fibrosarcoma	3.1	6.7	7.1
Undifferentiated sarcoma	3.1	4.0	3.6
Other malignant tumours of dermal origin	1.7	2.7	-
Fibroma	7.3	9.3	10.7
Leiomyoma	1.7	4.0	-
Other benign tumours of dermal origin	2.8	4.0	3.6
Sub-Total	88.1%	88.0%	75.0%
Unknown pathology	6.3%	6.7%	17.9%

The data on the cumulative incidence of skin tumours as a function of time after uniform irradiation showed that the latent period was a function of dose up to 100Gy, but that at doses above 100Gy, up to 1000Gy, tumours appeared 20-40 weeks post-irradiation, irrespective of dose level.

Data below 300Gy are best fitted by a linear induction and simple sterilisation function: $De^{-D/Do}$ and the high

incidence at the lowest (20Gy) dose precludes any threshold model. This lack of a threshold in the response is underlined by the results of the second series of experiments with doses of 2, 5, 10 and 20Gy and figure 2 shows the combined uniform dose response relationship. This has a p value of 0.1 and a turnover dose point of 220 ± 60Gy.



A linear regression analysis of the data below 150Gy, constrained to pass through the origin by the method of least squares, fitted well and explained 97% of its variability, the goodness of fit confirming the lack of threshold.

The data in fig.1 clearly indicate a reduced tumour incidence, at all doses, for the non-uniform irradiation, with the most marked reductions seen with the more inhomogenous, 8-point source.

II. Thulium-170 beta carcinogenesis in different mouse strains.

The strain difference experiment is almost completed, however, the analysis of the data is still preliminary, although nevertheless interesting.

The two albino strains, CD1 and SAS/4, both show a firm dose response relationship in their cumulative tumour incidence. The CD1 strain has a higher response at all dose levels, but also has a 17.5% tumour incidence in the control group, in contrast with the SAS/4's zero control incidence.

The C57Bl/6 black strain show an interesting response with a possible threshold effect. None of the control mice produced a tumour on the irradiated area and at the lowest dose level of 12.5Gy, one animal produced at tumour at 124 weeks post-irradiation; the remaining dose groups appear to have identical dose response curves with a peak cumulative tumour incidence at 120 weeks post-irradiation of 30-40%.

The CBA brown strain produced no tumours in the control group and a single tumour at the lowest 12.5Gy dose level. There was a strong dose response relationship in the cumulative tumour incidence at the higher doses.

A full pathological analysis is still to be completed. To date, the majority of the tumours have now been classified as malignant fibrous histiocytomas (MFH's). Also, it appears that the pigmented mice appear to be less susceptible to the induction of malignant epidermal tumours than are the albino strains, but these conclusions are still preliminary.

III. Curium-244 alpha carcinogenesis data.

To date, after 12 to 24 months post-alpha irradiation, with doses between 2Gy and 180Gy, no skin tumours have been recorded in the 760 SAS/4 mice in the study.

Discussion.

The literature on experimental radiogenic skin cancer studies has recently been reviewed (Coggle and Williams, 1990). Albert, Burns and their colleagues have carried out a large number of experiments in CD strain rats using X rays, monoenergetic electrons, protons and alpha and beta particles (see Burns and Albert, 1986). The dose effect relationship for electrons was curvilinear and tended to approach linearity for high LET radiations and at high doses, the incidence of tumours gradually declined.

A similar dose response pattern, of a curvilinear increase with dose for both epidermal and dermal tumours with a considerable fall-off at the highest doses, was reported by Hulse (1967) in CBA mice following thallium-204 beta irradiation. In more recent papers, using the same beta

sources, the incidence curves for both types of tumour showed little tendency to fall in mice for doses as high as 260Gy (Hulse et al, 1983). Finally, Tanooka (personal communication, 1984) found no skin tumours in the dorsal skin of female CD mice given single doses of strontium-90 beta irradiation up to 175Gy, and more recently, Ootsuyama and Tanooka (1988) have reported threshold doses for tumour induction following chronic or promoted ^{90}Sr irradiation.

In humans, there appears to be a racial variation in the incidence of amelanotic skin cancers induced by ionising radiation. About a dozen studies of irradiated human populations have been reviewed by Shore (1990) and it is of note that they provide no evidence for a dose threshold and are compatible with a linear dose response relationship. A figure of approximately 10^{-4}-10^{-5} cm^{-2} Gy^{-1} is obtained from these epidemiological studies and if these skin cancer risks are applicable to the whole body skin area then the skin cancer risk for the whole body could be as high as 10-100% Gy^{-1}.

The studies reported in this paper add to the sparse literature on experimental skin cancer induction. The uniform/ non-uniform thulium-170 experiments (fig.1) clearly offer no evidence for any mechanism giving rise to a 'hot particle effect'; the data refute any such hypothesis. These thulium experiments confirm the work of Albert and Burns who showed that sieve and grid patterns of exposure afforded a degree of protection against skin tumour induction.

The detailed pathological analysis of approximately 400 radiogenic skin cancers in our mice showed 4% of epidermal origin, 96% of dermal origin and this is comparable with results in CBA mice as found by Hulse et al (1983) and in contrast with 95% epidermal : 5% dermal in the rat (Burns and Albert, 1986). However, in contrast to Hulse's CBA mouse data, the SAS/4 dose responses (fig.1 and 2) do not indicate any evidence for a threshold and this is in agreement with the totality of the human radiogenic skin cancer data currently being reviewed by the ICRP Skin Task Group. The dose response (fig.1) shows no tendency to decline at high doses indicating that any cell-killing component must be minimal.

Our failure so far to induce any skin tumours using the 8cm² curium alpha source is not surprising since its depth dose characteristics preclude any significant alpha penetration of the critical dermal structures that give rise to 96% of the radiation induced tumours in the beta experiments described above.

The intercomparison of the carcinogenic response in different strains of mice following between 12.5 and 100Gy showed some wide variations in incidence, latency and tumour spectrum. For example, the minimum latent period appears to be not only dose related but also related to the longevity of the particular strain, such that despite producing the same overall numbers of tumours, the minimum latent periods for the CBA strain were much longer than those of the SAS/4, the latter being the shorter-lived. This may throw an interesting light on the problems of extrapolating data from the short-lived rodent to the human. These data also support the theory that even within a single species, variations in the target cell populations will be critical in determining cancer proneness.

Despite the differences between experimental results, useful inferences about the temporal and dose responses can be drawn by comparing the number of tumours per cm² of skin induced in mice and rats with the human data. This comparison is discussed in full in our recent paper (Coggle and Williams, 1990). Suffice to report here that the human skin cancer incidence is some two orders of magnitude lower than the totality of the rodent data, the latter showing a surprising degree of convergence. This discrepancy may be because human tumours are almost wholly epidermal, of extremely long latency and are widely under-reported, whereas in our experience of experimental systems, epidermal tumours form only a small percentage of the radiation induced tumours.

The most important message of this longterm study of non-uniform beta exposure of mouse skin is that the results are in direct contradiction to the 'hot particle hypothesis' and indicate that non-uniform exposure produces far fewer tumours than uniform exposure. This should be reassuring to those in

radiation protection who are currently concerned about the biological effectiveness of such particulates.

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Other research group(s) collaborating actively on this project [name(s) and address(es)]:

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Publications:

(1) Skin Carcinogenesis following Uniform and Non-uniform Beta Irradiation. M.W. Charles, J.P. Williams and J.E. Coggle. Health Physics 55 399-406 1988.

(2) Skin Carcinogenesis in SAS/4 mice following beta irradiation. J.P.Williams, J.E. Coggle, M.W. Charles and J. Wells in 'Radiation Protection - Theory and Practice' (IOP Publishing Ltd) 463-466 1989.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor:

Contract no.: BI6-C-058-F

Commissariat à l'Energie
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Title of the research contract:

Problems related to skin and underlying tissues after accidents involving local irradiation. Experimental study in the pig.

List of projects:

1. Problems related to skin and underlying tissues after accidents involving local irradiation. Experimental study in the pig.

Title of the project no.:

Problems related to skin and underlying tissues after accidents involving local irradiation. Experimental study in pig.

Head(s) of project:

Dr F. DABURON

Scientific staff:

Drs D. HOFFSCHIR, J.L. LEFAIX, M. MARTIN and J. REMY

I. Objectives of the project:

Diagnosis, prognosis and treatment of acute localized irradiations in pigs. Dosimetric assays by non invasive biophysical methods to evaluate the size and intensity of the radiolesions as an aid for surgery. Pathogenic studies of irradiated tissues by histological, immunocytochemical and histoenzymological methods. Pharmacological treatment screening in pigs and rabbits. Surgical treatment in pigs. Post-irradiation fibrosis studies in pigs : this healing process is investigated with respect to extracellular matrix synthesis, cellular proliferation abilities and molecular biology aspects.

II. Objectives for the reporting period:

-Improvement of non invasive biophysical methods for the diagnosis of the location and the intensity of the radiolesions. As far as prognosis is concerned it is important to determine, as early as possible, the limits for tissue which will undergo necrosis and delayed fibrosis.

-Treatment assays with different classes of pharmaceutics: steroidal and non steroidal anti-inflammatory drugs; vasotropic substances, haemorheological agents (anti-ischemic), platelet anti-agregants and anti-histaminic drugs.

-Persistance of the fibrotic activity beyond normal healing time suggested that fibroblasts involved in this process would present some peculiar properties which were to be investigated: on one hand biochemical and cellular culture characteristics of the fibrotic fibroblasts were studied, as were in the other hand, their kariotypes and their molecular biology patterns.

Experimental models for localized irradiation studies (all doses are given at 2 cm depth i.e. 4 times higher at the skin basal layer):

Large White pigs, 40-60 Kg, were irradiated on the external side of the right thigh (m. biceps femoralis) with a collimated source of 192Ir at doses between 30 and 84 Gy.

New Zealand rabbits were irradiated with the same device on the back (m. iliospinalis) at doses between 10 and 40 Gy.

III. Progress achieved:

III-1. Diagnosis

a) Thermography: 6 pigs (3-30 Gy, 3-64 Gy). Combined thermographic measurements carried out with superficial probes (10 GHz microwave probe or thermistor) and a 3 GHz microwave probe for deeper measurements were able to discriminate between superficial and deep tissue injuries after local irradiation. Although the superficial thermic reactions (observed for the first ten days) were similar in both cases, the deeper thermic reaction was significantly higher (60-100%)in the case of deep radiolesions.

b) X Rays Computed Tomography(XRCT) and Nuclear Magnetic Resonnance Imaging (NMRI) : 10 pigs (4-84 Gy, 6-64 Gy), 36 rabbits (between 10 and 40 Gy).

The early oedematous reaction following local irradiation was visualized either by XCRT or NMRI.

Oedema made the oedematous tissues less dense for X-rays absorption and involved a higher proton density conspicuous with NMR imaging.

In these conditions it was possible, as soon as 3-4 days after irradiation to delimit tissue areas exposed to doses above 40-50 Gy in pigs and in rabbits. Blind NMR image analysis, pixel by pixel, for signal intensity and heterogeneity allowed a discrimination between rabbits exposed on the back at 10, 20, 30 and 40 Gy within 8 days.

c) Gamma scintigraphy: 12 pigs (3-30 Gy, 9-60 Gy).

Local scintigraphies were performed in pigs with 201 Tl chloride and a 99m Tc labelled glycolipidopeptide. After local irradiation (60 Gy) 201 Tl made obvious the early inflammatory reaction, followed by a latent period before a second wave of uptake by the radiolesion, which began towards the 30th day and plateaued from the 70th until the 200th day after irradiation (latest experimental data).

Using a 99m Tc labeled glycolipidopeptide, supposed to reveal activated macrophages, made obvious a significant uptake of the radiolesion which began towards the 50th day, reached a maximum between 70-90 days and decreased rapidly beyond.

This kinetic aspect of the radiolesion labeling suggested two hypothesis according to the pathological evolution previously studied: 201 Tl might label the fibroblasts and 99m Tc labeled glycolipidopeptide the macrophages. Then it might be possible to follow with these two markers the early hyperemic reaction, the inflammatory reaction with migrating macrophages around tissues proceeding to necrosis process and finally the replacement of necrotized tissues by fibroblasts, 201 Tl chloride being possibly the marker of fibrosis.

d) Biochemistry.

Histochemistry : 10 pigs (64-84 Gy).

Extemporaneous histological observations (for instance during surgery) were able to characterize as soon as on day 6 tissues exposed above 20 Gy where fibrinogen extravasation is obvious; by the 15th day deposit of fibronectin on the one hand and changes in energy metabolism enzymes on the other were evident in tissues irradiated above 30-35 Gy (threshold dose for muscular necrosis in our hands). At that time and for these doses not any modification could be seen by classical histological methods.

Serum biochemistry : 40 pigs (30-40-64-84 Gy).

Changes in acute phase proteins and muscular enzymes were studied in serum of irradiated and control pigs; some of the 30-40-64 Gy irradiated pigs were treated with anti-inflammatory or/and platelet inhibitor drugs.

Daily analysis of the evolution of serum albumin and globulins levels made it possible to discriminate between pigs irradiated at 84-64 Gy and those irradiated at 40-30 Gy. In the first group a close relation was observed between clinical, thermal and biochemical evolution of the pathological process. In the second group, change in the serum biochemistry was significant during the silent phase (from the clinical and thermal point of view). In both groups, rises of C.K., L.D.H. and myoglobin level were consistent with an important muscular necrosis development.

As we observed it previously for the thermal reaction, treatments acted in any case like a factor reducing irradiation doses with regard to biochemical profiles in serum.

Three peaks of serum interferon were found in the 84 Gy irradiated group (10 pigs),

corresponding to three inflammatory periods: first (24-48 h) during early erythema; second (10-14th day) during the epidermolysis and sloughing phase where a local inflammatory reaction was obvious at the periphery of the lesion; the last period correspond to massive necrosis with local inflammatory reaction and oedema (35-60th day).

III-2. **Treatment.**

a) Surgical treatment: 8 pigs, 64 Gy.

We pointed out previously the benefit of early removal (before day 10) of tissues given more than 30 Gy after local irradiation, on the extent of the radiolesions .

Removal of the irradiated skin only, in the first days after exposition, decreased largely the local inflammatory reaction and limited the spreading of fibrosis.

b) Medical treatment: 200 rabbits, 10-40 Gy.

A clinical long term study (40 weeks) was performed in 60 rabbits irradiated on the back with doses from 10 to 40 Gy; the dose of 20 Gy was choose for drug screening, considering both clinical and histological results.

In a second experiment 7 pharmacological treatments were given for 8 weeks to 140 rabbits exposed to 20 Gy on the back: steroidal (SAI) and non steroidal (NSAI) anti-inflammatory agents, 2 vasodilatators (VD), 1 platelet anti-agregant (PAG), 1 haemorheological agent (anti-ischemic) (HA) and a combination of NSAI + HA.

--Results:

-Anti-inflammatory agents (SAI and NSAI) had a limitating effect on the clinical evolution of the lesions, especially the cutaneous necrosis.

-Vasotropic substances (HA, VD, PAG) had no effect on the clinical evolution of the radiolesions and might exibit a rebound effect after the end of the treatment.

-Platelet inhibitor and haemorheological agent had no effect on the clinical evolution of the skin lesions but limited the deep muscular necrosis.

-Combination of HA and NSAI involved a dose reduction factor of about 2 with regard to the clinical evolution of the skin injuries and the muscle radionecrosis.

III-3. **Conclusion on diagnosis and treatments.**

- Diagnosis.

In our experimental model, the different methods described above made possible the discrimination between irradiation doses involving either a spontaneous healing process (30-40 Gy) or a permanent ulcer (64-84 Gy). Furthermore a threshold could be assessed for a muscular exposition between 20 and 30 Gy.

A major interest of these biophysical, biochemical and biological methods was to make obvious , and to a certain extent to quantify, the effects of different treatments as dose reducing factors.

- Treatments.

Surgical treatments trials in pigs led us to define two threshold for muscle radiosensitivity: about 30-40 Gy for direct necrosis and 10-20 Gy for the limits of delayed fibrosis extent in spontaneously healing radiolesions. After early removal of irradiated skin the fibrosis extent is limited to tissue given above 25-30 Gy.

As far as medical treatment is concerned, the combination of non steroidal anti-inflammatory and haemorheological agents, given for 8 weeks after exposition to 20 Gy irradiated rabbits, involved a dose reduction factor of 2.

III-4. **Fibrosis studies.**

- Objectives.

Post-irradiation fibrosis exhibited, particularly in non spontaneously healing lesions, a tendency to spread out in surrouding tissues, weakly or not irradiated; fibroblasts isolated from radiation-induced fibrotic tissues exhibited in culture an abnormal and activated phenotype. The first question about that cellular activation was related to the initial events of fibrogenesis : have the cells involved in the healing process been irradiated and subsequently retained cytogenetic anomalies ?

The second question was related to the late stages : which were the stimulatory factors responsible for the chronic inflammation in the fibrotic tissues ? Those factors could be of two types : either the products of degradation of the extracellular matrix, which are known to be stimulatory factors for fibroblasts; or different cytokines involved in inflammatory reactions, such as TNF (tumor necrosis factor) and IL-2 (interleukine 2). Furthermore other growth factors such as FGF (fibroblast growth factor β) and TGF (transforming growth factor) are known to regulate fibroblast proliferation and matrix synthesis.

- Methodology.

Fibrotic tissues were removed from the pig thigh lesion between 1 and 24 months after irradiation and fibroblasts were isolated and put into culture medium. Total RNA was extracted both from the tissues and from fibroblasts culture.

- Results.

In fibrosis tissue samples taken off after 3-5 months total protein synthesis (assessed with labeled precursor) was about ten times higher than in normal muscle : the collagen percentage was respectively 54 and 7 % .

Collagen synthesis was 20 times higher than in muscular tissue : ARNm levels were 8 times higher for type I collagen and 5 times higher for type III. Nevertheless, probably due to transcription ratio and ARNm half-lives, collagen III was more abundant in fibrotic tissues.

In fibrosis extracted fibroblasts (FEF) cultures fibronectin synthesis was 3-6 times higher than in normal dermal fibroblasts (DF); glycosaminoglycanes synthesis was 1.25 (dermatane sulfate, chondroitine) and 2.2 (hyaluronic acid) times higher. Collagen synthesis was 2 times higher in FEF with an ARNm transcription ratio increased for type III collagen and decreased for type I : the isotypes ratio (I/III) was 3 times lower than in DF.

As far as FEF proliferation in culture was 10 times higher than DF (even in 20 months post-irradiation samples) the possible role of growth factors was to be investigated in radiation induced fibrosis processes :

-the response of FEF to EGF (epidermal growth factor) assessed by increasing the cells number in culture was 2 times higher than for DF.

-a fragment of heparin degradation products (CY 222, Lab. Choay, France) was added to the cultures medium. That fragment modulated the abnormal collagen phenotype of the FEF, and particularly decreased the type III and V collagen synthesis.

-the number of cell receptors for the FGF was high on FEF cell membrane : 70,000 high affinity and 140,000 low affinity receptors.

-the expression of TGF β , TNF$^{\alpha}$and IL-2 were investigated at the mRNA level in both tissue and cell cultures. Preliminary data showed a high level expression of IL-2 receptors in the FEF.

- Conclusion.

The first results in cytogenetic studies suggested that FEF derived from irradiated cells. The high level of chromosome damages might be responsible for the pretransformed state of those fibroblasts and perhaps might lead to secondary radio-induced cancers. A consistent observation about this hypothesis was the spontaneous perennisation of FEF cell lines (at the present time more than 50 subcultures) when normal DF cultures died after 10-15 subcultures.

In vitro modulation of the collagen phenotype by the heparin fragments showed that the degradation products of the extracellular matrix might be regulatory factors of the fibrotic processes. As regards growth factors, their expression was studied as a function of time after irradiation, in order to understand the initial reactions in fibrosis genesis and the persistance of chronic inflammation in later stages; preliminary data showed that IL-2 and FGF could be involved in those processes.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

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-Laboratoire de Biochimie du Tissu Conjonctif, G.R. C.N.R.S. 40, Institut Universitaire de Recherche sur les Maladies Vasculaires. Faculté de Médecine, 8 rue du Gal Sarrail, 94010 Créteil : Prof. L. Robert.

V. Publications:

1 /

- 1985

- Daburon F., Lefaix J.L., Rémy J., Fayart G, Tricaud Y.
Intérêt et limites des mesures thermographiques microondes pour le diagnostic et le pronostic des irradiations aigues localisées chez le porc. Radioprotection, 1985, 20, 3, 207-225

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- 1986

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Proceedings of a Workshop Held in Saclay 1985,
British Journal of Radiology, 1986, suppl.N°19, 109-113

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Proceedings of a Workshop Held in Saclay 1985,
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Proceedings of a Workshop Held in Saclay 1985,,
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- **1987**

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- **1988**

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- **1989**

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2 /

- **1985**

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Evolution des lésions après irradiation aigüe localisée chez le porc. Essais de traitement médical et chirurgical.Rapport CEA-IPSN-DPS-84-05-SPE, 1985.

- **1986**

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- **1987**

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- **1988**

- El Nabout R., Martin M., Rémy J., Robert L., Lafuma C.
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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-C-059-I

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Title of the research contract:

Radiation damage and recovery of the immune system.

List of projects:

1. Age-related changes in immunological functions in relation to radiation exposure.

Title of the project no.: 1

Changes in immunological functions in relation to radiation exposure.

Head(s) of project:

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Scientific staff:

Dr. L. Adorini
Dr. D. Frasca

I. Objectives of the project:

1. Recovery of T cell functions in irradiated mice by immunoregulatory molecules.

2. Characterization of the interactions between the T cell receptor, antigenic epitopes and MHC class II molecules.

II. Objectives for the reporting period:

1. Restoration of T helper cell activity and IL-2 production in irradiated mice by injection of synthetic molecules:

1.1. thymosin alpha-1
1.2. hu r IL-1 beta
1.3. 163-171 nonapeptide of hu IL-1 beta

2. Mapping of antigenic epitopes that interact with MHC class II molecules and the T cell receptor

III. Progress achieved:

Expression of effector immune functions is modulated by cellular and molecular interactions in a complex immunoregulatory network. The antibody response of B cells to immunogens is induced by the stimulation of antigen-presenting cells (APC) and modulated by T cells and factors which regulate antibody quality (isotype, allotype and idiotype) as well as intensity and duration of the response. Following immunization antigen is phagocytized and processed by APC (macrophages, B cells, dendritic cells), and the resulting peptides are presented in association with MHC class II molecules to activate T helper (Th) cells. Activated Th cells express cytokine-specific receptors, including IL-2 receptors (IL-2R), produce and secrete several lymphokines (IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IFN-gamma), and trigger the activation of antigen-stimulated B cells to antibody production. Cytokines play a major role in amplification of the immune response. Thus, antigen-stimulated macrophages synthesize and release IL-1 that amplifies IL-2 production by antigen-activated Th cells. IL-2 promotes proliferation of activated T and B cells and modulates IL-2R expression, while other lymphokines regulate T and B cell differentiation.

Our studies in BDF1 mice on the radiosensitivity and recovery of unprimed Th cells have indicated that these immunoregulatory cells are very radiosensitive and recover slowly, as some functional defects are still detectable 3 months after 200 cGy and complete recovery is reached 6 months after 400 cGy. Recovery of Th cell activity from radiation damage is accelerated by injecting mice with synthetic thymosin alpha-1, a peptide of 28 aminoacid residues identified in bovine thymus extracts and very effective in the induction of T cell markers and functions. We found that a single injection of 10 ug of thymosin alpha-1 in 3 month old mice, 4 days after 200 cGy completely restores Th cell activity. Th cells were induced by carrier-priming 7 days after irradiation and titrated 4 days later by the ability of spleen cells from the carrier-primed mice to induce a hapten-specific antibody response of spleen cells from unprimed mice upon in vitro stimulation with the hapten-carrier conjugate.

Since irradiation is known to impair not only Th cell activity but also IL-2 production, experiments were performed to determine whether the injection of thymosin alpha-1 in irradiated mice could restore IL-2 production. Three month old mice were total-body exposed to 100, 200, 300, or 400 cGy of X rays and given a single injection of 10 ug thymosin alpha-1 1 hour, 4, 7, or 14 days after irradiation. Spleen cells were harvested 3 days after thymosin alpha-1 injection and assayed for IL-2 production upon in vitro stimulation by Concanavalin A and Phorbol Myristic Acetate. Results from this series of experiments show that IL-2 production is reduced by irradiation and

may be restored to some extent by a single injection of thymosin alpha-1, the recovery being more pronounced when thymosin alpha-1 is given early after small radiation doses. In a subsequent series of experiments mice exposed to 100, 200, 300, or 400 cGy were given 3 injections of 1, 10, or 100 ug thymosin alpha-1 1 hour, 1 and 2 days after irradiation. Spleen cells were harvested the day after the last thymosin alpha-1 injection and assayed for IL-2 production. Results from two experiments indicate that maximum recovery of IL-2 production is attained by 3 injections of 10-100 ug thymosin alpha-1 after 100-300 cGy (100%) or 400 cGy (55-70%). Furthermore, the proliferative response of mitogen-stimulated spleen cells from irradiated mice to exogeneous IL-2 added to culture is increased by thymosin alpha-1 injection, implying enhanced expression of IL-2R.

The results of these experiments altogether indicate that thymosin alpha-1-induced enhancement of Th cell activity in irradiated mice may be mediated by increased IL-2 production and expression of IL-2R. The effect of thymosin alpha-1 in irradiated mice may result from acceleration of T cell precursor maturation and/or expansion of the mature T cell population. These findings on the recovery of Th cells, IL-2 production and IL-2R expression are very promising as they clearly indicate that thymosin alpha-1 is a powerful immunoregulatory molecule that may be successfully used for treatment of T cell immunodeficiencies in accidentally or therapeutically irradiated persons. Such a conclusion might also be extended to other thymic hormones.

Immunogerontology has clearly demonstrated in animals and humans that the immune system undergoes profound changes with aging which favour infections, autoimmune diseases, and tumour development. Not only the proportion of the various cell populations changes with advancing age but also the radiosensitivity of these cells may increase as a result of the decreased level of enzymes that neutralize free radicals. It is therefore very relevant to radiation protection to study changes in immune functions of aging animals for precise evaluation of radiation-induced dysfunctions which cannot be readily extrapolated from radiobiological studies carried out in young-adult animals.

At variance with previous studies, the radiosensitivity of the immune system has been investigated on mouse populations exposed to sublethal doses of X-rays in adulthood or much later in aging. In our study the radiosensitivity of Th cells has been assessed in BDF1 mice exposed to 50-400 cGy at 3 or 18-22 months of age. Th cells were induced by carrier-priming 7 days after irradiation and titrated 4 days later. In each experiment Th cell activity of spleen cells from

irradiated mice was expressed as percent of the activity of spleen cells from unirradiated controls. The log residual Th cell activity as a function of radiation dose was fitted by a straight line forced through the origin and the D37 ± S.E. were calculated from each linear regression. Preliminary experiments indicate the following D37 ± S.E.: 225 ± 25, 241 ± 35, 557 ± 118 for young mice and 115 ± 12 for old mice. Thus, the radiosensitivity of Th cell activity appears 2-3 fold increased in old mice. These findings suggest the need for extensive studies of the immunological effects of irradiation in aging animals since the mean life span of the human population in Europe is continuously increasing and a progressively larger number of old individuals is exposed to the risk of accidental (non-professional) or medical irradiation.

In spite of its potent immunostimulatory effects, the possible use of IL-1 as immunomodulator in humans is hampered by its inflammatory effects. IL-1, indeed, induces a series of typical inflammation-associated metabolic responses, such as fever and prostaglandin G release from cells of hypothalamic thermoregulatory centers, neutrophilia, synthesis of hepatic acute phase proteins, and alteration of glucose homeostasis as well as of the blood levels of divalent cations and corticosterone.

In studies directed at defining possible functional domains within the IL-1 molecule, a synthetic nonapeptide of human IL-1 beta (VQGEESNDK, position 163-171) was identified which could mimic several of the in vitro and in vivo immunostimulatory activities of the entire protein but devoid of IL-1 like inflammatory effects.

In our studies the immunorestorative capacities of human recombinant (hu r) IL-1 beta and of its synthetic 163-171 fragment were compared in mice immunodepressed by aging, sublethal irradiation, or both. Injection of hu r IL-1 beta into immunodepressed mice immediately after carrier-priming could restore to normal level Th cell activity. The ability of spleen cells from hu r IL-1 beta-treated immunodepressed mice to produce IL-2 upon in vitro mitogen stimulation was also increased significantly as compared to that of untreated mice and approached that of immunocompetent controls. The immunorestorative activity of hu r IL-1 beta on Th cell activity and IL-2 production could be mimicked by the synthetic 163-171 nonapeptide which, at the doses used, produced in most instances even greater effects than the whole protein. Although the optimal immunorestorative doses of the 163-171 peptide were several orders of magnitude higher than those of hu r IL-1 beta, the complete lack of IL-1 like inflammatory effects suggests that the synthetic fragment may be successfully used as immunomodulating agent in the therapy of T cell

immunodeficiencies.

Based on these findings we investigated the radioprotective effects of hu r IL-1 beta and of the synthetic 163-171 nonapeptide on the survival of lethally irradiated mice. BDF1 mice were injected with 2 ug of the nonapeptide or the whole protein and total-body X-irradiated with 850 cGy (LD 100/ 20), 20 hrs later. Both molecules were found to protect mice from radiation death, although the nonapeptide is half as effective as hu r IL-1 beta which yields about 80% survival. These preliminary observations suggest the possible use of the nonapeptide to prevent radiation injury with no toxic effects.

As soon as the central role of T cells in immunoregulation has been established, the molecular characterization of the antigen-specific T cell receptor (TCR) has become a major issue of investigation. In our laboratory the TCR was firstly studied on a cloned T cell lymphoma line, LH8-105, obtained by radiation leukemia virus-induced transformation of hen egg-white lysozyme (HEL)-specific mouse suppressor T cells.

The LH8-105 cell clone constitutively releases in culture supernatant products that specifically suppress the T cell-dependent proliferation, antibody response, and delayed-type hypersensitivity to HEL. LH8-105 cells were found to express on the cell surface dimers of 84 Kd composed of two 42 Kd subunits immunoprecipitated by rabbit antibodies directed against TCR constant region structures of helper and cytotoxic T lymphocytes. Thus, the overall structure of the TCR expressed by LH8-105 cells is similar to that described for helper and cytotoxic T cells. These studies have been extended to examine the rearrangement and expression of genes encoding the alpha and beta chains of the TCR. These suppressor T cells were found to exhibit alpha chain gene rearrangement, deletion of both alleles of the constant region (C) gene segment C beta 1 and rearrangement of the two alleles of C beta 2 when analysed by Southern blot hybridization. Restriction analysis suggested that the DNA rearrangement is beyond the second joining-region (J) minigene of the J beta 2 cluster. These results establish that at least some mouse suppressor T cell clones, like helper and cytotoxic T lymphocytes, rearrange and transcribe the genes coding for the alpha and beta chains of the TCR.

Subsequent studies in our laboratory were addressed to functional aspects of the TCR by analysing how Th cells recognize protein antigens as peptide-MHC complexes espressed on the surface of APC. Synthetic peptides of HEL have been used to analyse an immunodominant epitope for Th cells of the H-2d haplotype, which is contained in

the HEL sequence 107-116 and is recognized in association with I-Ed molecules. The immunodominance of this epitope in HEL-primed H-2d mice was demonstrated by the analysis of the T cell proliferative response induced by synthetic peptides covering the entire HEL sequence. Single residue substitutions at position 113, 114 or 115 were found to abrogate the ability of peptide 105-120 to activate Th cells. Substitutions at residues 113 and 115 affect T cell recognition but not the binding to I-Ed molecules whereas a substitution at position 114 profoundly impairs the capacity of the peptide to interact with I-Ed molecules. Thus, a strict correlation exists between binding of a peptide to MHC class II molecules and its immunogenicity, since a single substitution drastically reduces binding capacity and abolishes immunogenicity. This indicates that the MHC molecules are able to discriminate between very similar structures and may, in part, explain how the limited diversity of the MHC class II molecules expressed by any given individual could select relevant epitopes from among the almost endless variety of protein and peptide antigens. Analysis of the structural characteristics of peptide ligands interacting with I-Ed molecules lead to the conclusion that interactions between peptides and MHC class II molecules are usually very permissive since about 80-90% of single residue substitutions in the peptide molecule are acceptable. The specificity of the immune response, however, is maintained by T cell recognition which accepts only 10-20% of substitutions in a given peptide.

Since the formation of peptide-MHC complexes is very permissive, peptides with unrelated sequences are expected to compete for binding to the same MHC molecule, and this, indeed, has been shown to occur in vitro. We, therefore, examined whether such a competition could also regulate T cell responses in vivo. We found that a synthetic peptide corresponding to residues 46-62 of mouse lysozyme, although not immunogenic itself, effectively inhibits the priming for T cell responses when injected into mice together with immunogenic protein or peptide antigens. The inhibition observed strictly correlates with the capacity of the competitor to bind to the particular MHC molecule presenting the foreign antigen, and its extent depends on the molar ratio between antigen and competitor. Thus, the mouse peptide 46-62, which binds to I-Ak molecules, prevented priming for Th cell responses by foreign peptides such as HEL 46-61 or HEL 112-129, which also binds to I-Ak molecules. These and other observations from our laboratory on the immunodominance of T cell epitopes demonstrate the exquisite MHC specificity of in vivo competition between peptides generated during antigen processing in APC. It is, therefore, conceivable to modulate Th cell activation by interfering with the binding of antigenic peptides to MHC class II molecules. This could open the way to a rational treatment of autoimmune diseases and allograft rejection.

IV Other research group(s) collaborating actively on this project [name(s) and address(es)]:

V. Publications: 101 (A + B)

A: Full papers in scientific journals and congress proceedings, monographs.

B: Short communications.

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-C-061-D

Universität Ulm
Abteilung für Klinische
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Title of the research contract:

Impairment of the hemo-lymphopoietic cell system and its microenvironment by ionizing radiation. Pathogenesis of non-stochastic and neoplastic effects and conditions for a long term restoration.

List of projects:

1. Mechanisms governing the response of the hemopoietic system and defining its tolerance to partial body and inhomogeneous irradiation.
2. Radiation-induced damage to the stroma - a limiting factor for hemopoietic reconstitution.
3. Pathogenesis of late hemopoietic failure and proliferative disorders in hemopoietic/lymphopoietic cell systems as a consequence of protracted low level radiation exposure.

Title of the project no.: 1

Mechanisms governing the response of the hemopoietic system and defining its tolerance to partial body and inhomogenous irradiation

Head(s) of project:

Prof. Dr.rer.nat. W. Nothdurft and Prof. Dr. med. T.M.Fliedner

Scientific staff:

Dr.K.Baltschukat (1985-1988), Prof. Dr.W.Calvo (1985-1987), Dr.L. Kreja (1985-1989), Prof. Dr.W. Nothdurft (1985-1989), Dr.I.Seitz (1985-1986), Dr.K.-H. Steinbach (1985-1988), Dr.C. Werner (1985)

I. Objectives of the project:

It was the objective of this project to investigate in which way the tolerance of the hemopoietic system to an exposure of the body to ionizing radiation, i.e. its compensatory capacity and the regenerative potential is dependent on the fraction of the bone marrow surviving the irradiation and its localization within the body. The events that had to be investigated in detail were (a) the compensatory response of the hemopoietic cells in the non-irradiated marrow, (b) the seeding of circulating stem cells to the damaged bone marrow sites, and (c) the recovery processes in the latter.

II. Objectives for the reporting period:

The specific aims were to study the local events and the systemic control of the hemopoietic response and the regeneration after partial body irradiation and after exposures to a uniform as well as an inhomogeneous total body irradiation on the level of the progenitor cell compartments using canine experimental models. Based on determinations of the progenitor cells of the granulocyte-macrophage and the erythroid lineage, GM-CFC and BFU-E, respectively, the acute changes in the hemopoietic tissue and the functional status in a long term were determined and related to the blood cell concentration. As a prerequisite of these in vivo studies progenitor cell analyses had to be performed characterizing the normal state with respect to their concentration in various bones of the total skeleton and their cycling state. In addition, it was of particular interest to study their radiation response characteristics.

III. Progress achieved:

1. Methodology

(a) The irradiations of the dogs (beagles) were performed with 300 KVp X-rays (HVL = 4 mm Cu) at a dose rate of 0.065 Gy/min on the middle axis of their body. Homogeneous total body irradiation (TBI) was achieved by bilateral exposure. The dogs (n = 3) received a dose of 2.4 Gy (on the middle axis in tissue). Measurements of the bone marrow doses performed in different bone cavities by means of thermoluminescent dosimeters (TLD-100, 1 mm x 6 mm) resulted in values ranging from 1.5 Gy to 2.0 Gy depending on the location. In contrast, an inhomogeneous TBI was performed in other dogs (n = 6) by unilateral exposure only, i.e. they were irradiated with their left side directed to the radiation source. Under there conditions the values of the doses in the different bone marrow sites showed considerable differences, ranging from 0.9 Gy (right iliac crest) to 3.1 Gy (head of the left humerus) at the extreme. Calculations of the mean marrow doses resulted in values of 1.7 Gy respectively 1.8 Gy for the bilateral and the unilateral exposure. For the fractional survival of the GM-CFC in percent of the total GM-CFC numbers in the whole body rather similar values were determined for either exposure, i.e. 6.7 % for homogeneous TBI and 7.7 % for the unilateral exposure. Lower body irradiation (LBI) was employed as an approppriate model of partial body irradiation. For LBI the lower part of the body including the last lumbar vertebrae, the lower extremities and the tail was exposed bilaterally to 11.7 Gy, while the upper part of the body including the fourth lumbar vertebra, the upper extremities and the head, was shielded in a lead box. LBI involved the abrogation of approximately 28% of the total bone marrow mass. The dose generated in the shielded part of the body was found two orders of magnitude lower than the delivered dose. In all the irradiated dogs sequential determinations of several hematological parameters were performed within a period up to one year after the exposure. These included determinations of the blood cell counts, of GM-CFC in peripheral blood and different bone marrow sites, and BFU-E at certain occasions.

(b) The GM-CFc were assayed by means of a standardized agar culture-technique using serum from irradiated dogs (0.3 ml/ml) as a source of colony stimulating activity, (CSA) and irradiated blood leukocytes from a normal dog for enhancement of colony growth. For the determinations of BFU-E appropriate methods of collecting the bone marrow and suitable culture conditions had to be established. Bone marrow samples were obtained from up to 18 different bones using different methods. In some cases the bone marrow was scraped out from a resected piece of the humerus or a rib. Erythrocyte-poor cell suspensions were obtained from the bone marrow samples by either sedimentaton in the presence of dextran (1.5%) or by density gradient separation over lymphoprep (density 1.077 g/ml or 1.070 g/ml). Initially, a culture system was employed

consisting of methylcellulose and 20% to 30% fetal calf serum, additives such as transferrin and α - thioglycerol, erythropoietin (Epo) at 2-2.5 IU/ml and canine leukocyte-conditioned medium as a source of burst-promoting activity (BPA). In the course of the research work some methodological studies were performed in which several modifications were applied to improve BFU-E growth, or eventually to simplify the culture system. In addition, culture techniques were established allowing the growth of multipotential progenitor cells (Mix-CFC) from canine bone marrow.

(c) The radiation response characteristics of different types of progenitor cells from canine bone marrow were determined under conditions of in vitro irradiations with 280 kVp X-rays (HVL = 2 mm Cu) at a dose rate of 0.72 Gy/min. Values of D and n were calculated from the linear parts of the survival vs. dose plots on a semi-logarithmic graph by means of linear regression analysis.

2. Results

(a) Progenitor cell concentrations in the bone marrow under normal conditions.
In most bone marrow sites in the bones of the trunk-skeleton containing red marrow the GM-CFC concentration was found in the range from 180 to 350 per 10 mononuclear cells (MNC). The red marrow in the spongeous structures of the proximal part (epiphysis) of the humerus. contains GM-CFC at concentration falling in the upper range of the values. On the other hand, significantly lower concentrations were obtained for the marrow in the vertebrae. Rather low GM-CFC concentrations of approximately 70 per 10 MNC and 50 per 10 MNC were present in the proximal part of the femur and the distal part of the humerus, respectively. However, considerable differences could be detected between the GM-CFC levels in individual dogs of the groups under study.

Only small differences were observed between the BFU-E growth for MNC suspensions that were obtained from the marrow samples by sedimentation in the presence of dextran or by density separation using Lymphoprep of a density of 1.070 g/ml. However, colony formation could be increased by a factor of 1.5 if instead of 20% FCS a mixture of 20% FCS and 10% serum collected from total body irradiated (3.9 Gy) dogs at day 10 after the exposure was added to the cultures. Obviously, the acts as a source of BPA more effective than leukocyte conditioned medium. In any case, the BFU-E values determined in bone marrow samples from different sites were significantly lower than the GM-CFC numbers, i.e. between 43 and 118 per 10 MNC in comparison to between 180 and 350 for the latter in the same sites. Again there was the,

tendency for the humeral marrow to have higher BFU-E concentrations than the bone marrow of the iliac crest and the sternum. The BFU-E concentration in the peripheral blood was found to be in the range from 30 to 100 per ml and thus only slightly lower than the GM-CFC concentration.
On the other hand, the cloning efficiency of BFU-E in bone marrow cell suspensions could be increased by a factor of 2-4 if the cell separations were performed with Lymphoprep of a density of 1.070 g/ml instead of 1.077/g/ml. Furthermore, the colony growth of BFU-E in cultures containing fetal calf serum (FCS) or without FCS was much better when recombinant human Epo (rh-Epo) instead of the natural Epo from anemic sheep was used as a specific stimulator. Similar results were obtained for the canine multipotent progenitor cell Mix-CFC. All the other recombinant factors of human origin (rh-GM-CSf, rh-G-CSF, rh-M-CSF, rh-Il-3) exhibited no canine BFU-E stimulating activity. The factor rh-IGF-1 was found, potentiate the effect of BPA on BFU-E in suboptimally stimulated cultures.

(b) Radiation response of erythroid progenitor cells (BFU-E)

For the determinations of the radiation response characteristics of the BFU-E suspensions of bone marrow MNC were irradiated as indicated with increasing radiation doses. Thereafter, the cells were cultured in methylcellulose. The survival fractions obtained for BFU-E after irradiation under steady state conditions could be optimally fitted by a simple exponential curve S = exp with a value of Do = 0.15 Gy. Thus, the BFU-E were found extremely radiosensitive when compared to the GM-CFC, for which under similar conditions values of Do in the range from 0.55 Gy to 0.61 Gy were obtained.
Additional experiments were performed to test whether the radiosensitivity of the BFU-E could be influenced under conditions of enhanced proliferation. However, it was found difficult to establish appropriate culture conditions that would allow the BFU-E to proliferate in parallel to their differentiation. The best results with a recovery factor of 70% after 24 hs were obtained when the BFU-E were cultured in the presence of FCS and 1-2 IU Epo/ml. The results obtained with this method indicate that the radiosensitivity may decrease when the cells are proliferating in suspension cultures under optimal growth conditions.

(c) Irradiations of dogs under different exposure conditions

(α) Homogeneous total body irradiation by bilateral exposure (BLE)

The most important hematological effects of the acute TBI with a midline-in-tissue dose of 2.4 Gy can be summarized as follows: the lymphocytes had dropped to approximately 20 % of the initial average value within 24 hs after the exposure. The recovery was slow as expected and

at day 125 the lymphocyte concentration had just recovered to approximately 50 % of the normal values. The granulocyte concentration had reached its nadir at day 8 after TBI, when it had droppped to approximately 25 % of the pre-irradiation value. The definite granulocyte recovery commenced at day 20 and at day 40 the values were back in the pre-irradiation range. The GM-CFC in the different bone marrow sites had decreased to between 3 % and 7 % of their initial concentration. In all the bone marrow sites the recovery of the GM-CFC concentration was quite fast in the period between day 14 and day 28. However thereafter the GM-CFC values remained at a subnormal level, i.e. between 50 % and 86 % of the pre-irradiation values, up to day 125.The BFU-E concentration in the different bones was found extremely reduced 24 hs after the exposure to between 0.2 % and 3 % of the inital values depending on the site. These in vivo data are compatible with the high radiosensitibity of the BFU-E to in vitro irradiation, as compared to the GM-CFC. The BFU-E in the different bones showed a steady increase in the period between day 1 and day 28 after the exposure. However, the BFU-E values in all the bones remained subnormal up to 1 year after the exposure, and clearly these residual deviations from the pre-irradiation state were more pronounced than for the GM-CFC.

(β) Inhomogeneous total body irradiation by unilateral exposure (ULE)

The inhomogeneous TBI was characterized by the following radiation doses: entrance dose = 3.8 Gy, midline-in-tissue dose = 2.7 Gy, and exit dose = 0.9 Gy. As indicated under 1. Methodology, part (a), the extreme values of the bone marrow doses were 3.1 Gy for the cavity in the head of the left humerus and 0.9 Gy for the marrow cavity in the right iliac crest. However, the hematological effects of these irradiations conditions as observed in the blood leukocytes and the thombocytes were concruent with those as reflected in the dogs which had received the homogeneous TBI by bilateral exposure. Thus, with respect to the overall hematological consequences both BLE and ULE were isoeffective. Nevertheless, the inhomogeneous dose distribution in the various bone marrow sites was quite well reflected by the degree of reduction in their GM-CFC concentration at day 1 after the exposure with values ranging from 0.44 % to 16 % of the inital concentration, depending on the location in the body. At the same time the S-phase fraction of the GM-CFC was found significantly increased in different bone marrow sites.

The fast regeneration of the GM-CFC in the period up to day 28 was strictly dependent on the initial degree of the damage. On the other hand, the GM-CFC values remained clearly subnormal up to day 60 after the exposure; at day 125 they were rather normal again.

In the unilaterally exposed dogs the BFU-E concentration was determined in the bone marrow of the head of the humerus that was directed away from the source, i.e. was protected by the body and thus received less irradiation, i.e. 1.1 Gy and consequently less damage than most of the

other bone marrow sites. On the first day after the exposure the BFU-E had dropped to values between 1 % and 8 % of the initial concentration as predicted on the basis of their extremely low Do value. However, in the interval between day 1 and day 14 after irradiation the BFU-E concentration showed a rapid increase to between 150 % and 250 of the pre-irradiation value in contrast to the GM-CFC that within the same period had % reached only approximately 16 % of the pre-irradiaiton values. Thereafter the BFU-E remained in the normal range or remained slightly above up to 1 year after irradiation. In contrast, the GM-CFC values in the same bone marrow sites were found still subnormal at day 48 and day 60, but had normalized at day 125 at least.

( ) Partial body irradiation

The intensive irradiation with a dose of 11.7 Gy of the bone marrow in the lower part of the body corresponding to 30 % of the total bone marrow mass resulted in rather moderate transient changes of the blood cell concentration. The granulocyte values showed a transient fall to approximately 75 % of the pre-irradiation values and a complete recovery within the first 30 days after the exposure. The platelet concentration decreased to approximately 80 % of the average pre-irradiation level with the nadir at day 21. Thereafter it increased rapidly and was virtually back in the normal range at day 30. In contrast the lymphocytes showed a much stronger response insofar that they had decreased to 40 % of the pre-irradiation level already within the first 24 hs after the exposure. It took about 130 days before they had reached their normal pre-irradiation level.

The GM-CFC concentration in the protected bone marrow (humerus, scapula) showed an immediate decrease to approximately 40 % of the pre-irradiation values within 24 hs after the irradiation of the lower part of the body (LBI). This was followed by an increase in the following 30 days that led to normal values for the GM-CFC in the humerus but still clearly subnormal values for the GM-CFC in the scapula. The S-phase cells in the protected marrow increased by more than 100% within 1 day after the exposure and remained elevated until day 36.

In the irradiated bone marrow sites of the iliac crest no progenitor cells could be detected at the first day after the exposure. However, there was a rapid increase in the bone marrow GM-CFC concentration to 50 % of the pre-irradiation level by day 21. On the other hand, there was no further increase in the period up to day 60 after the exposure. Slightly higher values were found at day 140, but there was no complete recovery in the irradiated sites one year after irradiation. The GM-CFC in the blood showed a severe depresssion within the first 24 hs after the exposure. The subsequent course of events was characterized by a transient increase between day 10 and 25 with a second decrease in the period from day 30 to 45. At least at day 130 the GM-CFC in the blood had returned to the preirradiation levels.

III. Discussion

The survival of persons exposed to irradiation in the lower range of possibly fatal doses depends mainly on the functional status of the hemopoietic tissue and its regenerative capacity. This has been clearly shown during the recent accidents in nuclear power stations and industry. However, the anatomical distribution over the different bones of the hemopoietic stem cells and progenitor cells surviving an exposure may be quite different depending on the conditions of the exposure, i.e. whether it was total body irradiation with a homogeneous bone marrow dose distribution or a more or less inhomogeneous dose distribution. Under extreme conditions partial body irradiation may arise with certain fractions of the whole bone marrow organ being completely or nearly absolutely protected against the incident beam.

Under the former conditions the regeneration events may be mainly local originating from resident surviving stem cells. However, it has to be asked whether after an inhomogeneous total body irradiation the local regeneration of the different bone marrow sites (receiving different degrees of damage) is associated with systemic equilibration processes due to exchanges of hemopoietic cell migration via the circulation. Clearly, after partial body irradiation where large doses in the range of 10 Gy had been received by a certain fraction of the marrow seeding of stem cells from the protected marrow could be the most essential prerequisite for any early repopulation. On the other hand in the early phase of regeneration the protected marrow may compensate in an essential way for the damage in the early phase of regeneration.

The research work performed in the present project was addressed to all these aformentioned aspects of hemopoietic regeneration and tolerance due to compensatory mechanisms.

The results obtained in our normal dogs from the quantitative determinations of the GM-CFC and BFU-E indicate that the concentration of each of these progenitor cells in different bone marrow spaces containing active red marrow is not uniform. On the other hand, there is obviously a close correlation between the concentration of the GM-CFC and BFU-E in the various bone marrow sites. In either case the differences may become important under conditions of inhomogeneous or partial body irradiations and at least have an influence on the early response of the granulocyte renewal system.

Both types of canine progenitor cells the GM-CFC as well as the BFU-E are inactivated by irradiation in vitro in a strictly or quasi-exponential fashion; and the same holds true for the multipotential progenitor cell Mix-CFC. This is in general accordance with most data obtained for progenitor cells from human bone marrow, when the colony growth is stimulated by conditioned media from different sources. However, to our knowledge the canine BFU-E seems to be the most radiosensitive cell among all the hemopoietic elements from the different species.

Nevertheless, the results obtained from the BFU-E determinations in the dogs which received a homogeneous TBI indicate that the BFU-E and the GM-CFC in identical bone marrow sites may have rather different repopulation kinetics after certain exposure conditions. The BFU-E showed a steady sharp increase in the period from day 1 to day 28. However, up to 1 year after TBI with a dose of 2.4 Gy they remained clearly more suppressed in relation to the pre-irradiation levels than the GM-CFC.

The data obtained on the first day after the inhomogenous TBI from the comprehensive GM-CFC determinations in 7 different bone marrow sites are in accordance with the response predicted on the basis of the bone marrow dose distribution. Consequently, the actual surviving fractions may be a valuable indicator of the radiation damage received by a certain amount of bone marrow. In this context it is of special interest to notice that the GM-CFC survival fractions in different bones of the unilateral by irradiated animals at the extreme varied by a factor of approximately 36. Nevertheless, it could clearly be shown that despite the inhomogeneous dose distribution the integral (or systemic) damage to the bone marrow of these dogs was similar to that obtained in the dogs which received the homogeneous TBI by bilateral exposure. Namely, that both exposures were hematologically isoeffective became evident from the congruencies in the changes of the blood cell concentrations of all lineages. Thus, using this canine model it could clearly be demonstrated, that the degree of the blood cell depression and its temporal pattern of changes is determind by the total fraction of surviving clonogenic hemopoietic cells independent of the pattern of their distribution within the skeleton.

Several conclusions of theoretical as well as of practical interest can be drawn from the data obtained using the canine model of partial body irradiation. First of all it could be demonstrated that the exposure of 30% of the total bone marrow mass resulted in a severe lymphocyte depression within 24 hs after the exposure, whereas the blood granulocyte and platelet values showed only minor changes within the first 3 weeks following the exposure. Therefore, an initial lymphocyte depression after irradiation may well be a monitor per se that in fact certain damage has been caused; however, the lymphocyte changes cannot be applied as a prognostic indicator of hemopoietic recovery. The reason for this is that in the case of partial body irradiation the protected bone marrow whether 70% or 30 % of the total mass is able to compensate effectively for the damage in the exposed sites by rapid cell proliferation and differention within 24 hs after irradiation. On the other hand, it could be shown that in the highly irradiated bone marrow sites there was a significant degree of repopulation by hemopoietic elements already at day 7 after the exposure. This fact is clearly due to the seeding of stem cells from the protected marrow sites since in dogs receiving homogeneous TBI with a much lower dose of 3.9 Gy progenitor cells could be detected at much lower numbers if any at day 7 or 9 after the exposure.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

Our Ulm research group is an active member of the European late Effects Project Group, in which 19 European research laboratories "compare notes" on these irradiation studies.

V. Publications:

1. Original publications, reviews, book articles

Baltschukat,K., and W. Nothdurft (1990)
Hematological effects of unilateral and bilateral exposures of dogs to 300 kVp X-rays.
Radiat. Res. (in press)

Baltschukat, K., W. Nothdurft and T.M. Fliedner (1989)
Hematological effects in dogs after irradiation of the lower part of the body with a single myeloablative dose.
Radiother. Oncol. 14, 239 - 246

Fliedner, T.M. (1988)
Hematological indicators to predict patient recovery after whole-body irradiation as a basis for clinical management.
International conference: The medical basis for radiation accident preparedness: II. Clinical experience and follow-up since 1979, Oak Ridge, 19-23 October, 1988

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In: Zhang Q-X, Wu D-C (eds.) Radiation biological effects. Modifiers and treatment. Proceedings of the International Conference on Biological Effects of Large Dose Ionizing and Non-Ionizing Radiation, Hangzhou, March 29- Avril 1, 1988. Society of Radiation Medicine and Protection, Chinese Medical Association, Beijing, pp 60-91

Fliedner, T.M., T. Szepesi and K.H. Steinbach (1987)
Medizinische Versorgung der vom Reaktorunfall Tschernobyl unmittelbar betroffenen Personen.
In: Niklas, K., W. Börner, F. Holeczke und O. Messerschmidt (Hrsg.) : Tschernobyl und die Folgen. Begutachtung von Strahlenschäden [Strahlenschutz in Forschung und Praxis Bd. 29], pp 85-103, Gustav Fischer Verlag,Stuttgart, New York, 1987

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Growth of erythroid burst-forming units (BFU-E) in cultures of canine bone marrow and peripheral blood cells: effect of serum from irradiated dogs.
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In vitro studies of the sensitivity of canine bone marrow erythroid burst-forming units (BFU-E) and fibroblast colony-forming units (CFU-F) to X-irradiation.
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In: Scherer, E., C. Streffer and K.-R. Trott (eds): Radiophathology of organs and tissues. [Medical Radiology - Diagnostic Imaging and Radiation Oncology]. Springer Verlag, Berlin, Heidelberg, New York, (in press)

Nothdurft, W. (1990)
Use of peripheral blood stem cells for transplantation - experimental protocols performed by the Ulm Group.
Review, International Conference on the occasion of the 60th Birthday of T.M. Fliedner, Schloß Reisensburg, Sept. 28 - October 1, 1989, (in press)

Nothdurft, W., K. Baltschukat and T.M. Fliedner (1988))
Untersuchungen über die Kompensationsmechanismen und Regeneration der Hämopoese nach einzeitiger Halbkörperbestrahlung im Tierexperiment an Hunden.
[Invited Paper, European Symposium 1987 on Half Body and Total Body Irradiation, Sept. 30 - October 3, 1987, Dresden/DDR] Radiobiol.- Radiother. 29, 334-336

Nothdurft, W., W. Calvo, V. Klinnert, K.H. Steinbach, C. Werner and T.M. Fliedner (1986)
Acute and long-term alterations in the granulocyte/macrophage progenitor cell (GM-CFC) compartment of dogs after partial-body irradiation. Irradiation of the upper body with a single myeloablative dose.
Int. J. Radiat. Oncol. Biol. Phys. 12, 949-957

Szepesi, T., T.M. Fliedner and K.H. Steinbach (1988)
Hematological responses after accidental exposure to ionizing radiation: a review of 22 reported accidents.
In: Zhang Q-X, Wu D-C (eds.) Radiation biological effects. Modifiers and treatment. Proceedings of the International Conference on Biological Effects of Large Dose Ionizing and Non-Ionizing Radiation, Hangzhou, March 29 - Avril 1, 1988, Society of Radiation Medicine and Protection, Chinese Medical Association, Beijing, pp 30-59

2. Short communications, abstracts

Baltschukat, K., L. Kreja, W. Nothdurft and W. Weinsheimer (1989)
Acute and long-term alterations of erythroid burst forming units (BFU-E) in dogs after totalbody irradiation.
Paper, 22nd Annual Meeting of the European Society for Radiation Biology, September 11-16, 1989, Brussels, Book of Abstracts, p. 127

Baltschukat, K., W. Nothdurft and T.M. Fliedner (1987)
Acute and residual haematological effects of partial body irradiation of dogs: I. Irradiation of the lower part of the body with a single myeloablative dose of 11.7 Gy.
Int. J. Radiat. Biol. 51, p. 747 [Abstract]

Baltschukat, K., W. Nothdurft and T.M. Fliedner (1987)
Unilateral exposure of dogs to 300 kV X-rays: hematological effects of an inhomogeneous dose distribution assessed by granulocyte/macrophage progenitor cell (GM-CFC) determinations.
8th International Congress of Radiation Research, 19.-24.7.1987, Edinburgh, Abstracts part E, No. E4o-8V, p. 284

Title of the project no.: 2

Radiation damage to the stroma - a limiting factor for hemopoietic reconstitution?

Head(s) of project:

Prof. Dr. W. Nothdurft, Prof. Dr. Calvo (1985)

Scientific staff:

Prof. Dr. T.M. Fliedner, Prof. Dr. H.J. Seidel, Dr. V. Klinnert (1985), Dr. K.H. Steinbach (1985-1988), Dr. B. Heinze (1985-1989), Dr. L. Kreja (1986-1989)

I. Objectives of the project:

The various cell types forming the cellular stroma (reticulum cells, endothelial cells, fibroblasts/fibrocytes, macrophages, nerve fibres) and its extra-cellular matrix are considered rather resistant to radiation with respect to acute destruction. However, due to their long lifespan late disintegration of the stroma is of importance resulting in functional and structural alterations that again may lead to bone marrow hypoplasia or even aplasia. Therefore, the sensitivity of the stroma in situ and certain cell types to ionizing radiation and its impact on the hemopoietic supportive function was studied using in vivo and in vitro assay systems.

II. Objectives for the reporting period:

The specific aims were to define and characterize the stromal elements with respect to their biological and biophysical properties and their capacity to support hemopoietic function under normal conditions and after irradiation. For this purpose both in vitro and in vivo studies were performed using mainly canine models. In vitro certain stromal cells were studied with respect to their clonal growth characteristics and proliferative capacity and their radiation response characteristics. Suspension cultures prepared from fetal organs with their unique hemopoietic activity were employed to eventually produce specific hemopoietic growth factors for further characterization. At least long-term co-cultures had to be established with the aid

to analyze short-range interactions between the stroma and hemopoietic elements. In vivo the association between the different types of stroma cells and the different hemopoietic cell lineages was studied in specimens from different tissues of the fetus and the adult. The models of partial body irradiation and inhomogeneous total body irradiation were employed to study hemopoietic reconstitution in relation to irradiation-induced stromal changes. At least as another approach to the same problem transplantations of hemopoietic cells from fetal liver were performed into recipients which before had been treated with high dose TBI.

III. Progress achieved:

1. Methodology

(a) For in vitro studies of different stromal functions and analyses of certain cell types cell cultures were prepared from bone marrow aspirates or biopsy material from different bones of normal adult dogs or from different fetal organs showing hemopoietic activity. The fibroblast/fibroblastoid colony forming cell (CFU-F) was taken as representative of clonogenic progenitor cells of the hemopoietic supportive stroma. Their biological and radiobiological properties were determined under different conditions. This included systematic studies to be performed to find out the optimum sampling techniques, separation methods and cell culture conditions. Various approaches to establish long-term canince bone marrow cultures were based on the culture system according to Dexter. First, different bone marrow preparations were used to establish an adherent cell layer in the culture flasks as a hemopoietic supportive microenvironment. Second, different culture conditions especially with respect to the serum components in the medium were tested for their influence on the maintenance of hemopoietic activity. In addition, the influence of several growth factors was tested by means of progenitor cell determinations.

(b) The radiation response characteristics of the CFU-F were determined under conditions of in vitro irradiations with 280 kVp x-rays (HVL = 2 mm Cu) at a dose rate of 0.72 Gy/min. Values of D and n were derived from the linear parts of the survival vs. radiation dose plots on a semi-logarithmic graph by means of a linear regression analysis. Colony scoring was performed at day 14 after the exposure.

(c) Different types of radiation exposures using moderate to high radiation doses were employed to study the in situ effect of irradiation on the stroma in general and its progenitor cell compartment CFU-F in particular. Bone marrow specimens including pieces obtained from the ribs were used for determinations of the total cell number and of the CFU-F and the granulocyte-macrophage progenitor cell (GM-CFC). In addition, qualitative and quantitative histological and electronmicroscopical techniques including histochemical approaches were applied to the exminations of the microenvironment and the associated hemopoietic elements.
The irradiations of the dogs (beagles) were performed with 300 kVp X-rays (HVL = 4 mm Cu) at a dose rate of 0.065 Gy/min on the middle axis of their body. Homogeneous total body irradiation (TBI) was given as a conditioning regimen to dogs which thereafter received transplantations of fetal liver cells for hemopoietic reconstitution. The dogs were irradiated by bilateral exposure with a total dose of 17.5 Gy given in 3 fractions of 5.85 Gy each within 5 days. An

inhomogeneous TBI in a much lower dose range was given to other dogs and was achieved by unilateral exposure (ULE), i.e. the dogs were irradiated with their left side directed to the radiation source. Accordingly the values of the doses in different bone marrow sites showed considerable differences ranging from 0.9 Gy (right iliac crest) to 3.1 Gy (head of the left humerus) at the extreme. Partial body irradiation was performed as upper body irradiation (UBI). The upper part of the body including the head and the fourth lumbar vertebra was exposed bilaterally to a single dose of 11.7 Gy, while the lower part of the body including the lower extremities and the tail was shielded in a lead box. Sequential partial body irradiation was given to other dogs to study the regeneration of the hemopoietic tissue in a highly irradiated stroma and its compensatory response to a second challenge. In this case the animals first received bilateral upper body irradiation (UBI) with a dose of 11.7 Gy and after 56 days they received lower body irradiation (LBI) with the same dose.

2. Results

(a) Stroma progenitor cells CFU-F

Bone marrow was collected from normal dogs using different methods and several cell separation techniques were applied to find out the optimum sampling conditions for CFU-F. Bone marrow aspirates from which the cell suspensions for culturing were prepared by sedimentation in the presence of dextran (1.5%) or by density separation over Lymphoprep (density 1.077 g/ml) yielded extremely low numbers of CFU-F. In contrast, if the cell suspensions were separated using the buffy coat method by simple centrifugation a cloning efficiency of 40 to 90 CFU-F per 10 bone marrow cells was obtained for aspirates from different bones. Similar values were obtained for marrow samples that were scraped out from resected bones and cultured without further separation. Interestingly, using the same methods between 3 and 5 CFU-F per 10 bone marrow cells only were found in the fatty marrow in the head of the tibia. The low numbers correlated quite well with the low incidence of hemopoietic progenitor cells in the same site. The cells defined as CFU-F were found to be rather heterogeneous with respect to cell size (as determined by velocity sedimentation) and did not show the capability of phagocytosis.

Reproducible survival data for canine bone marrow CFU-F were obtained in all of the experiments performed. Obviously survival was related to the radiation dose not in a simple exponential fashion. An appropriate fit to the empirical data was obtained by means of a multitarget-model $S = 1-[1-\exp(-D/D\ )]n$. However, the survival fractions showed a clear negative slope in the range of doses below 1 Gy. The values of the survival curve parameters obtained for CFU-F from the humerus were $D = 2.61 \pm 0.40$ Gy, $n = 1.04 \pm 0.42$, and from the iliac crest $D = 2.41 \pm 0.38$ Gy, $n = 1.38 \pm 0.62$.

The capacity of CFU-F for repair of sublethal damage after in vitro irradiation was tested in the following way using the split-dose technique: The cells were kept in suspensions at appropriate concentrations and irradiated at room temperature with increasing doses in the range from 0.36 Gy to 9 Gy. Cell suspensions that had received a dose of 2.9 Gy were kept at either room temperature or 37°C for 2 hs to allow possible recovery from the first dose and then were again exposed to incremental doses to establish a survival curve. The survival data obtained for the CFU-F after the second irradiations were characterized by the following features: Generally the CFU-F number had decreased 2 hs after the first dose to approximately 60% of the original survival fraction independent of the incubation temperature in the interval between the two fractions. However, the slopes of of the survival curves obtained after the second irradiations were rather different from the single-dose data. Thus, there is in principle some recovery from sublethal damage of the CFU-F. However, in the test system employed it was macked by interfering effects perhaps due to suboptimal holding conditions in the interval between the exposures.

(b) Liquid cultures of cell suspensions from fetal tissues

Experiments were performed in which cell suspensions prepared from different fetal organs were kept in liquid cultures in order to investigate, whether or not, and if, under which circumstances the cells would be able to produce factors stimulating hemopiesis. It could be shown that fetal liver cells (2 x 10 cells/ml) taken from a 35 old canine fetus were able to produce factors that were stimulating megakaryocyte formation in vitro. Interestingly, in the primary cultures osteoblasts and osteoclasts were found among the adherent cell layers.

(c) Long-term bone marrow cultures

Different bone marrow preparations were tested for their suitability to establish adherent cell layers. The best results with a maintenance of the layer over more than 10 weeks were obtained when bone marrow was scraped out from the bones and cultured directly without any further preparation, or when bufty coat cells were used collected from bone marrow cell suspensions after centrifugation at 400 g for 10 min. On the other hand, nucleated cell suspensions obtained from bone marrow aspirates by means of sedimentation in the presence of dextran or by density gradient centrifugation (Lymphoprep) produced poor layers if any. The maintenance of hemopoietic activity in the cultures as assessed by the presence of GM-CFC was clearly dependent on the serum constituents of the culture medium and further supplementation. At least GM-CFC could be found up to 8 weeks after preparation of the cultures, when the culture medium was supplemented with dog and horse serum (10% each) and 10 mol hydrocortisone. No further improvement could be achieved with culture flasks that were prepared with an extracellular matrix.

In contrast to bone marrow cultures of canine cells long-term cultures with hemopoietic activity for approximately 13 weeks could be established for murine cells on the basis of the Dexter system. Up to this time the presence of GM-CFC as well as pluripotent stem cells (CFU-S) could be demonstrated. It was interesting to notice that in the murine bone marrow cultures adipocytes (fat cells) were quite frequent in contrast to the canine bone marrow cultures. Some improvement in the hemopoietic activity in the latter could be achieved when dog serum containing colony stimulating activity or conditioned medium from allogeneic bone marrow cell suspensions was added to the long term cultures.

(d) Effects of irradiations under different exposure conditions

(α) Fractioned total body irradiation followed by fetal liver transplantation The CFU-F numbers in pieces of the ribs of a defined length of 1 cm were determined in dogs which had received fractionated TBI with a total dose of 17.5 Gy and transplants of fetal liver cells thereafter. The CFU-F numbers remained clearly subnormal up to 2 years after treatment. On the other hand, hemopoietic recovery was quite fast when compared to transplants of canine bone marrow cells, and no significant hemopoietic defects could be observed. This raises the question as to the significance of determinations of CFU-F numbers as a monitor of the hemopoietic supportive capacity of an irradiated stroma.

(β) Inhomogeneous total body irradiation

Under the conditions of the unilateral exposure employed quite different radiation doses were obtained in the bone marrow of the ribs on the left side of the body or the right side, i.e. 2.7 Gy and 1.1 Gy. The most essential findings obtained 1 year after the exposure were as follows: The number of the CFU-F obtained for the bone marrow of the left rib was by a factor of x 0.71 lower than that obtained for the right side. Yet, this difference was insignificant. In contrast, the total number of hemopoietic cells determined as mononuclear cells (MNC) in total or as GM-CFC was significantly lower in the bone marrow of the left ribs that had received 2.7 Gy when compared to the contralateral ribs that had received 1.1 Gy.

(τ) Upper body irradiation

It was of interest to notice that in the dogs which had received a single dose of 11.7 Gy given to their upper body the numbers of CFU-F in the ribs had decreased to less than 20% of the pre-irradiation value in the period up to day 80 after the exposure. At least one year after

the treatment there was a gradual return toward normal of the CFU-F numbers in the bone marrow of the ribs. Using histological techniques and cytological criteria it could be shown that the cellularity in the irradiated bone marrow sites was clearly reduced up to day 120 at least and was nearly normal again at day 380. The stroma showed an increase in the number of connective tissue fibres, especially in areas attached to the bone trabeculae. In areas in which fibrosis was marked there was little or no hemopoietic activity.

(δ) Sequential hemibody irradiation

To study whether an irradiated stroma is able to support a compensatory response of the hemopoietic tissue to an extraordinary challenge the hematologic changes were studied in dogs which received first a single myeloablative dose of 11.7 Gy to their upper body and, after an interval of 56 days were given the same dose to their lower body. The bone marrow volumes irradited during the first and the second exposure were equivalent to approximately 70% and 30%, repectively, of the total bone marrow mass. At day 56 after the exposure of the upper body, when the second irradiation (lower body) was performed the granulocyte values were still slightly subnormal. At the same time the GM-CFC concentration in the different irradiated bone marrow sites, i.e. the humeri, the sternum, the scapulae and the ribs, had recovered to between 27% and 40% of the normal values. In all the pre-irradiated bone marrow sites in the forepart of the body a slight compensatory increas in the GM-CFC was already observed at the first day after the exposure of the lower body. The increase in the GM-CFC concentration continued up to day 21. It was related to a significant rise in the Sphase fraction within the same period of time. The maximum GM-CFC values were followed by a second decrease and a levelling-off within the lower range of the inital values or at quite normal levels beyond day 120 after the last exposure.

3. Discussion

The structural integrity of the bone marrow stroma and its functional status is the essential prerequisite for the maintenance of hemopoiesis and its regenerative and compensatory capacity under disturbed conditions. Thus, it is of importance to study the biology of the stroma and its elements under normal circumstances and the effects of different types of exposures to ionizing radiation on the stroma itself.

One approach in the present programme was to study the fibroblastoid colony forming unit (CFU-F). These cells are considered to be representative of the progenitor cell population of the hemopoietic microenvironment. Consequently, they might be of importance in a longterm for the replacement of the long-lived differentiated elements of the stroma, such as fibrocytes, reticulum cells etc.

The results obtained indicate that the concentration of the CFU-F in different bones is closely related to the hemopoietic activity in the respective bone marrow sites as measured by the incidence of hemopoietic progenitor cells, i.e. GM-CFC and BFU-E. With respect to their radiobiological properties it is of interest that the clonogenic CFU-F are relatively radioresistant when compared to hemopoietic progenitor cells. The D values of 2.4 Gy of the canine bone marrow CFU-F are by a factor of 4 higher than those for the GM-CFC, and they are obviously by a factor of approximately 2 higher than the D values reported for human bone marrow CFIU-F. The presence of a shoulder, though quite small, on the survival curve is indicative of some capacity for accumulation of sublethal damage. Therefore, some capacity of sublethal damage repair could also be expected. However, when split-dose irradiations were performed a significant loss of CFU-F after the first dose could be noted. Perhaps this loss was due to the fact that the CFU-F were irradiated in suspension and not in an adherent state. Nevertheless, when the survival data were corrected for the loss a clear split-dose recovery became evident if the two doses were separated by an interval of 2 hs.

In the dogs which had received inhomogeneous TBI by an unilateral exposure the differences that could be detected between the CFU-F numbers in the ribs at least 1 year after the exposure were rather small. These findings show that the initial cell loss and the differences in the CFU-F numbers must have been nearly compensated within the 1 years period.

On the other hand, there was clearly some late damage present in the hemopoietic tissue in the bone marrow that had received a dose of 2.7 Gy. This was obviously not directly related to the CFU-F.

The data obtained from the partial-body irradiated dogs clearly indicate that certain types of damage to the stroma are responsible for the delayed regeneration of hemopoiesis as reflected in the subnormal GM-CFC values in bone marrow sites that had received a single dose of 11.7 Gy. It could be shown that the CFU-F numbers in the irradiated sites also remained clearly subnormal for several months after the exposure. On the other hand, in this model microscopical examination revealed structural alterations in the stroma evident as an increased number of connective tissue fibres. Clearly, in areas in which fibrosis was marked there was little or no hemopoietic activity. However, despite such local defects the hemopoietic function in a given site was nearly normal after 1 year as became evident from the GM-CFC determinations in aspirates.

Sequential half body irradiation was employed to test whether a pre-irradiated stroma is able to allow a compensatory response of the hemopoietic tissue similar to that in an unirradiated state. It could be demonstrated that bone marrow sites that had partially recovered from a radiation dose of 11.7 Gy were able to compensate effectively for the damage that was produced in other parts of the skeleton by a second irradiation with the same dose, if this second irradiation was performed 56 days after the first one. The compensation was achieved in the same way as observed in a previously unirradiated bone marrow, namely by a strongly increased proliferation within the following 3 weeks. Thus it can be concluded that the stroma that had received a dose of 11.7 Gy is able to supply the microenvironmental conditions required for such an extraordinary response of hemopoiesis.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

These was an active collaboration within the European Late Effect Project Group (EEULEP) and its 19 research institutions.

V. Publications:

1. Original publications, reviews, book articles

Bödey, B., W. Calvo, O. Prümmer, T.M. Fliedner and M. Borysenko (1987)
Development and histogenesis of the thymus in dog. A light and electron microscopical study.
Dev. Comp. Im. 11, 227-238

Calvo, W., T.M. Fliedner, E. W. Herbst, E. Hügl and B. Bödey (1987)
Degeneration and atrophy of the thymus of lethally irradiatied dogs, rescued by transfusion of cryopreserved autologous blood leukocytes.
Exp. Hematol. 15, 1171-1178

Calvo, W., J. W. Hopewell, H.S. Reinhold, A.P. van den Berg and T.K. Yeung (1987)
Dose-dependent and time-dependent changes in the choroid plexus of the irradiated rat brain.
Brit. J. Radiol. 60, 1109-1117

Fliedner, T.M. and K.H. Steinbach (1988)
Repopulating potential of hematopoietic precursor cells.
Blood Cells 14, 393-410

Klinnert V, W. Nothdurft W and T.M. Fliedner (1985)
CFU-F from dog marrow: a colony assay and its significance.
Blut 50, 81-87

Kreja, L, K. Baltschukat and W. Nothdurft (1988)
Growth of erythroid burst forming units BFU-E in cultures of canine bone marrow and peripheral blood cells. Effect of serum from irradiated dogs.
Exp. Hematol. 16, 647-651

Kreja, L., K. Baltschukat and W. Nothdurft (1989)
In vitro studies of the sensitivity of canine bone-marrow erythroid burst-forming units (BFU-E) and fibroblast colony-forming units (BFU-E) and fibroblast colony-forming units (CFU-F) to X-irradiation.
Int. J. Radiat. Biol. 55, 435-444

Michel, C., W.Calvo, A. Raghavachar and T.M. Fliedner (1986)
Histochemical studies on the effects of lethal total body X-irradiation on the pancreas of dogs rescued by autologous bone marrow transplantation
Cell. Mol. Biol. 32, 519-526

Michel, C., W. Calvo, O. Prümmer and T.M. Fliedner (1987)
Histochemical studies on the pancreas of dogs rescued by fetal liver cell transplantation after lethal total body X-irradiation.
Cellular and Molecular Biology 33, 91-100

Nothdurft,W., K. Baltschukat and T.M. Fliedner (1989)
Hematological effects in dogs after sequential irradiation of the upper and lower part of the body with single myeloablative doses.
Radiother. Oncol. 14, 247-259

Prümmer, O., W. Calvo and T.M. Fliedner (1987)
Variation of treatment conditions alters the outcome of fetal liver transplantation.
Thymus 10, 19-31

Prümmer O., Raghavachar A, Fliedner T.M. (1985)
Recovery of immune functions in dogs after total body irradiation and transplantation of autologous blood and bone marrow cells.
Exp. Hematol. 13, 891-898

Prümmer O., A. Raghavachar, C. Werner, W. Calvo, F. Carbonell, I. Steinbach, T.M. Fliedner (1985)
Fetal liver transplantation in the dog. I. Restoration of hemopoiesis with cryopreserved fetal liver cells from DLA-identical siblings.
Transplantation 39, 349-355

Raghavachar A., O. Prümmer, W. Calvo, W. Nothdurft, K.H. Steinbach, T.M. Fliedner (1985)
Repopulating potential of canine bone marrow cells: differences between large and small cells separated by velocity sedimentation.
Brit. J. Haematol. 60, 33-40

2. Short communications, abstracts

Fliedner, T.M. (1987)
Experimentelle Grundlagen der Stammzelltransplantation.
Deutsche Gesellschaft für Innere Medizin, Bd.93, 66-75

Fliedner, T.M., W. Nothdurft and W. Calvo (1986)
Bone marrow repopulation and extramedullary hemopoiesis in dogs after partial body irradiation.
Exp. Hematol. 14, p. 460 [abstract]

Fliedner, T.M., W. Weinsheimer, W. Nothdurft and W. Calvo (1988)
Bone marrow structure in its significance for the regulation of hematopoiesis.
XXII Congress of the International Society of hematology, Milano, August 22-September 2, 1988. Book of abstracts, p. 155

Kreja, L., K. Baltschukat and W. Nothdurft (1988)
In vitro studies on the X-ray radiosensitivity of canine bone marrow erythroid burst forming units (BFU-E) and fibroblast forming units (CFU-F).
3. Symposium Molekulare und zelluläre Mechanismen der biologischen Strahlenwirkung München-Neuherberg, 23.-25. März 1988. Book of abstracts.

Kreja, L., K. Baltschukat and W. Weinsheimer (1989)
Long-term radiation effect on bone marrow BFU-E and hemopoietic regeneration in total body irradiated dogs.
4. Jahrestagung der Deutschen Gesellschaft für Hämatologie und Onkologie, 1.-4. Oktober 1989; Hannover, Blut 59, abstract no. 72

Kreja, L., W. Nothdurft and K. Baltschukat (1987)
Long-teerm canine bone marrow culutre.
Meeting of the European Stem Cell Club, April 29-30, 1987, Institute Pasteur, Paris, Book of Abstracts, Abstract No.33

Nothdurft, W., K. Baltschukat and T.M. Fliedner (1987)
Acute and residual haematological effects of partial body irradiation of dogs: II. irradiation of the uper and lower part of the body with single myeloablative doses separated by an interval of 56 days.
Int. J. Radiat. Biol. 51, 252 [Abstract].

Title of the project no.: 3

Pathogenesis of late hemopoietic failure and proliferative disorders in hemopoietic/lymphopoietic cell systems as a consequence of protracted low level radiation exposure.

Head(s) of project:

Prof. Dr. T.M. Fliedner and Prof. Dr. H.J. Seidel

Scientific staff:

Prof. Dr. W. Calvo (1985-89), Dr. M. Gulich (1989), Dr. A. Ingendaay (1987-88), Dr. L. Kreja (1985-89), Prof. Dr. W. Nothdurft (1985-89), Prof. Dr. H.J. Seidel (1985-1989), Dr. K.-H. Steinbach (1985-1987), Dr. L. Weber (1988-89), Dr.Dr. D. Zinser (1988-89)

I. Objectives of the project:

It was the objective of this project to analyse the pathogenetic mechanisms that eventually lead to the development of myelogenous leukemia or to aplastic anemia when mammals are being exposed to ionizing radiation or cytotoxic drugs. In addition, it was of interest to study the effects arising from the combined action of radiation and certain cytotoxic chemicals.

II. Objectives for the reporting period:

Several experimental models and modes of treatment were employed depending on the specific aspects to be studied.

In mice, the problem was studied in what way the total body irradiation as a co-carcinogen could enhance the leukemogenic effect of the drug methylnitrosourea, that specifically causes T cell leukemias. The leukemogenic effect of benzene after inhalation as a consequence of chronic exposure to low concentrations of the compound was also studied in mice. In these experiments certain analyses of early stem cell changes were performed. However, they were mainly directed to long-term effects with respect to the induction of malignancies.

In dogs the progenitor cell changes and other hematological parameters were examined during and after continuous treatment with low doses of intravenously injected cyclophosphamide as a model of chronic damage to the hemopoietic cell compartments. With respect to hematological radiation effects in man retrospective analyses of peripheral blood cell changes in persons as a consequence of (accidental) protracted radiation exposure were performed.

III. Progress achieved:

1. Methodology

a) In the studies of the leukemogenic effects of methylnitrosourea (MNU) and total body irradiation female BDF1 mice, 4 or 10 weeks old, were used. MNU was dissolved in saline and administered intravenously. Total body irradiation was performed with 280 kV X-rays (filter 1.5 mm Cu and 1 mm Al) at a dose rate of approximately 0.3 Gy/min. The irradiation was given in various doses in single sessions or fractionated, and at different intervals before or after MNU. The bone marrow and the spleens of mice were used to prepare cell suspensions for hemopoietic stem cell and for natural killer cell assays.
In the past 2 years the methodology used in these murine leukemogenesis studies was further developed by establishing molecular biology techniques such as transfection, Southern and Northern blotting in close collaboration with the European Late Effects Project Group.

b) The studies on the leukemogenic effect of benzene were performed using an inhalation chamber that allowed chronic exposure of the mice to defined low concentrations of gaseous benzene. The mice were exposed to different concentrations of benzene for periods up to 8 to 16 weeks.

c) In beagles, the studies on the hematological effects of protracted treatment with cyclophosphamide were performed as follows: cyclophosphamide (Cy) was given at a dose of 3 mg/kg body weight per day for 23 days. Several hematological parameters were determined, but special emphasis was given to the determinations of the granulocyte/macrophage progenitor cells (GM-CFC) in the bone marrow and the blood. A biomathematical model developed to simulate canine granulopoiesis was employed for these data also.

d) As far as the human hematological data from radiation accidents is concerned, detailed information about the Algerian accident became available (and later, a continuously updated data bank on radiation accidents was established). In the Algerian accident 4 persons were involved receiving total body irradiations at doses in the range from 8.62 to 10.15 Gy during 38 days. Thus the daily dose amounted up to 0.23 - 0.27 Gy. This exposure resulted from a 192-radium source. In all these patients a severe granulocytopenia was observed at the end of the exposure. The patients received intensive medical treatment, and spontaneous hemopoietic recovery commenced around 35 days after the end of the exposure.

2. Results

a) Leukemogenesis in mice

The major question was whether there is a synergistic action of the sequential treatment of the mice with total body irradiation and the drug MNU. Furthermore, it had to be asked whether an impaired NK cell function was involved. With a given dose of 50 mg/kg of MNU an "optimal" leukemia (T cell-lymphoma) induction with various mouse strains and also mice in various ages was found, the median induction time varied around 18-20 weeks, more than 95% of the animals died from malignancy. Additional external radiation in doses of 12 x 0,1.25 Gy (in weekly intervals during the first period of the latency period) to 12 x 0.75 Gy had no influence on these endpoints. There was however, as expected, a significant influence on hemopoietic stem cells. With 50 mg/kg of MNU also single doses of radiation either before or after the chemical were not effective with respect to leukemia development. There were, however, two experimental conditions, that reveal a synergistic effect. When the NMU dose was reduced, with the consequence of a prolonged latency period, repeated doses of radiation did reduce the latency period to the above mentioned 18-20 weeks. Single doses of radiation between 1.5 and 4.5 Gy 2-5 weeks before lower doses of MNU (40 mg/kg) did also enhance the leukemia development. Natural killer cell activity could not be correlated to the leukemogenesis. Effects on the NK cells like injection of corynebacterium parvum, use of various mouse strains with other background activities, and also use of endotoxin or antithetaglobulin were without influence.
The model was then used for molecular analysis. DNA preparations were obtained from bone marrow and thymus during the latency period and used for transfection. Some active preparations were found rather early (after 3-5 weeks), but so far the responsible onc-genes have not been identified. Since mutated N-ras genes are found in MNU induced leukemias, a series has been begun to look for these mutations using the polymerase-chain-reaction (in collaboration with Janowski/ Mol).

b) With benzene 30% malignancies of the lymphoid systems were observed in a longterm experiment. The hemopoietic stem cell system was carefully analysed during a benzene exposure period up to 16 weeks. The late erythroid stem cells (CFU-E) turned out to be the most sensitive parameter for the toxic action of this solvent. Adaptation of the erythroid system to this stress condition could be analysed - there is an enhanced turnover of erythroid precursors.

c) In cyclophosphamide-treated beagles the effects in the hemopoietic cell system as seen after low dose irradiation could be induced. The biomathematical model, developed for radiation, could also be used for an analysis of these changes, which occurred on the level of stem cells, cells in the peripheral blood and also regulatory factors (CSA).

d) The hematological data from this radiation accident were used to compare the kinetics of depression and recovery with the results of experimental protocols in dogs in order to improve the model. After this it was decided to build up a data base for observations in human beings after radiation exposure. This has led to a new large research project which evolved from this contract (Post-Chernobyl-studies).

Discussion

Hemopoietic failure (aplasia) and malignancy as a consequence of radiation exposure are difficult to analyse in their stages of development. Even in mice no clear indication for the most critical decision between the two endpoints aplasia or neoplasia could be found. Stem cell depression may and may not be involved, the NK cell system had no influence. In a given leukemogenic protocol a synergism of a chemical agent and external radiation was seen, when each agent alone was in a suboptimal dose. The enhancement of leukemogenesis in such protocols by preceeding radiation is thought to be a target cell phenomenon. During the recovery from a median dose of radiation more target cells seem to be present than under steady state conditions. - If mutations in the N-ras gene are a critical event, the more recently established methods of molecular biology should be able to detect mutated genes in various hemopoietic organs during the latency period. This will contribute to the question of the target cell system.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

There has been extensive exchange of data, mainly with the EULEP Laboratories, but also with E.P. Cronkite/Brookhaven. The molecular analysis is being done in a joint effort with the Department of Radiobiology in Mol.

V. Publications:

Fliedner, T.M., W. Nothdurft and W. Calvo: The development of radiation late effects to the bone marrow after single and chronic exposure.
Int. J. Radiat. Biol. 49, 35-46, 1986

Seidel, H.J.: Induktion von Leukämien - Indikator einer Ganzkörperbestrahlung.
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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-C-082-UK

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Central Elect. Generating Board
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Telephone number: 0453-810451

Title of the research contract:

The establishment of radiological protection criteria for non-uniform skin exposure.

List of projects:

1. A cooperative study to establish radiation protection criteria based on experiments on pig and mouse skin.

Title of the project no.:

A cooperative study to establish radiation protection criteria for non-uniform skin exposure based on experiments on pig and mouse skin.

Head(s) of project:

M W Charles

Scientific staff:

M W Charles
J Wells

I. Objectives of the project:

A collaborative programme will investigate non-stochastic effects in mouse and pig skin and stochastic effects in mouse skin, following uniform and non-uniform alpha and beta irradiation. This should enable radiological protection criteria to be developed for limiting non-uniform skin exposure and will provide the design parameters for personal skin dosemeters. In particular the aim is to identify the nature and depth of cells at risk in the skin and to identify an appropriate area over which dose should be averaged.

II. Objectives for the reporting period:

III. Progress achieved:

1. Introduction

The current recommendations of the International Commission on Radiological Protection (ICRP 1977) regarding limitation of skin exposure are on the basis of non-stochastic effects. An annual dose limit of 0.5 Gy is recommended on the basis of a life-time exposure limit of 20 Gy and a target cell depth of between 50-100 micrometers (μm) or 5-10 mg cm^{-2} mass thickness in tissue. Because of the paucity of human and animal data the ICRP have been unable to provide definitive advice regarding the appropriate area and depth over which this dose should be averaged, particularly for small areas and highly non-uniform exposures such as those from small radioactive sources ('hot particles'). Recently, however, considerable information on non-stochastic and stochastic effects following radiation exposure of the skin has become available from animal studies and recent epidemiological studies in man also provide new information on the risk of radiation induced skin cancer. In this paper this new information, with associated dosimetry data, will be combined in order to suggest how an improved, coherent and defensible system of skin dose limitation may be achieved.

2. Stochastic effects

The risk of skin cancer mortality given by the ICRP in 1977 was considered to be much less than that for other organs, and on this basis non-stochastic effects were considered to be the limiting biological end-point. The ICRP (publication 28) subsequently gave a mortality risk figure of 10^{-4} Sv^{-1} for a whole body skin exposure.

The recent data of Shore (1989) which are based on an exhaustive review of all the available human data, give a whole body radiation induced skin cancer risk figure. This amalgamates risks for those parts of the body subjected to UV exposure together with the significantly lower risk for the remaining parts of the body normally shielded by clothing from UV exposure. The average whole-body skin cancer risk figures, for both sexes combined, are given in Table 1 and support current ICRP risk figures. On the absolute model the risk is about half that given previously by the ICRP while on the relative risk model the risk is perhaps twice that previously given by the ICRP.

TABLE 1

RADIATION INDUCED SKIN CANCER RISK FIGURES FOR MAN

	Incidence/10^{-4} Sv^{-1}	Mortality*/10^{-4} Sv^{-1}
Absolute risk model	233	0.47
Relative risk model	971	1.94

* Using 0.2% mortality (data from Shore, 1989).
For comparison the 1978 ICRP risk figure for skin cancer mortality is 10^{-4} Sv^{-1}.

The choice of skin depth which is appropriate for evaluating skin dose relevant to skin cancer induction is a matter of some controversy. The observation of excess basal cell carcinomas in uranium miners subjected to alpha irradiation of the skin would seem to support epidermal involvement but some questions remain regarding the veracity of this particular study. On the basis of equivocal evidence it would be prudent to assume the potentially pessimistic use of the basal layer of the epidermis as the target cells for skin cancer induction.

2.1 Non-fatal cancers

Since the clinical treatment of skin cancer is relatively inexpensive and generally involves little trauma and only a few days of lost work, the question arises as to whether any detriment should be associated with successfully treated skin cancers.

A recent NRPB review of radiation risk philosophy (Clarke 1989) indicated that in the future it was likely that non-fatal cancers would receive a weighting in order to evaluate health detriment. The NRPB have in fact given whole body exposure risk figures based on an assumed detriment weighting (let us call this W_D) of 0.1-0.2 for non-fatal cancers. A high W_D value could over-ride the difficult choice of a figure for the proportion of skin cancers which prove to be fatal, which forms the basis of a skin cancer mortality risk figure (assumed to be 0.2% in Table 1).

2.2 Skin cancer following spatially non-uniform skin exposure.

In the case of highly non-uniform exposures, where some cells may be subjected to high doses, it is possible that the mean dose may underestimate the stochastic risk due to a supralinear dose response relationship, or over-estimate due to cell sterilisation. Some models have postulated that localised gross tissue damage may produce a dramatic increase in tumour incidence by several orders of magnitude compared to uniform exposures (the so called 'hot particle effect'). Evidence against this hypothesis has been found in studies of skin cancer induction in rodents following non-uniform exposures. In one such study (Charles et al 1988) mouse skin was subjected to a wide range of beta doses, down to 2 Gy (skin surface dose) using a plane uniform source of ^{170}Tm (Emax 0.97 MeV) or arrays of 8 and 32 small (2 mm diameter) sources distributed over the same area of 8 cm^2. For all doses (averaged over the total area of 8 cm^2) the highest tumour incidence occurred following uniform exposures. These data refute the 'hot particle' hypothesis and support the use of mean dose as a conservative measure of carcinogenic risk following spatially non-uniform skin exposure.

3. Non-stochastic effects

Non-stochastic effects were considered by the ICRP in 1977 to be more important than stochastic effects for skin dose limitation and they recommended an annual skin dose limit of 0.5 Sv (20 Gy life-time). In fact, few relevant data were available to the ICRP at the time to support this dose limit. More recent results have become available from this collaborative radiobiology study of the effects of beta irradiation on pig and mouse skin. The late dermal response in the two species differs due

to the difference in blood supply to the skin. Since the blood supply in pigs is similar to that in man these results have been used primarily as the basis for considerations relevant to radiological protection.

3.1 Acute tissue breakdown.

Dose effect curves have been obtained for acute tissue breakdown of pig skin for a variety of beta radiation source sizes. For these studies the skin surface doses were measured with an extrapolation chamber with an effective window thickness of 16 μm and an electrode area of 1.1 mm^2 (Wells 1988). The ED_{50} and ED_{10} values were calculated using probit analysis and are given in table 2. The pronounced dependence of effect on beta energy ($^{90}Sr/Y$, ^{170}Tm and ^{147}Pm: Emax:2.27; 0.97 and 0.225 MeV) and the influence of the radiation source size (0.1 - 40 mm diameter) in 3-4 month old female Large White pigs are clearly seen. The early epithelial responses were consistent with repopulation of the irradiated epidermis by surviving epithelial cells from around the hair follicle and from the periphery of the irradiated area. For $^{90}Sr/Y$ and ^{170}Tm larger sources produced moist desquamation which occurred within 4-5 weeks of exposure, following the mitotic death of basal epidermal stem cells. For smaller sources, after higher local doses, the tissue breakdown is due to interphase death of dermal cells, resulting in ulceration which occurs at about 1-2 weeks. For the lower energy ^{147}Pm sources an acute epidermal breakdown occurred at 1-2 weeks. This early and transient response to ^{147}Pm was due to interphase death of suprabasal epithelial cells subjected to very high doses. For the larger sources the doses required to produce epidermal breakdown in 50% of the irradiated fields (ED_{50}) are about 28, 73 and 360 Gy (measured at a depth of 16μm) for $^{90}Sr/Y$, ^{170}Tm and ^{147}Pm respectively.

It does not appear to be possible to choose a unique single area and depth over which to average dose which will provide a single predictive indicator for all source sizes and beta energies. This is presumably due to the various complex end-points actually involved in the acute responses of the skin. However, the use of an average dose over an area in the vicinity of 1 cm^2 does lead to considerable accord in the dependence of ED values on source size for the range of beta emitters used in these studies. Some limited extrapolation chamber measurements have been made, using various sizes of collector electrode, which enables some guidance to be given on the relationship between the doses from these sources when averaged over different areas (Wells et al 1982; Wells 1986, 1988). Table 3 provides information which indicates the ratio between the measured dose at 16 μm averaged over an area of 1.1 mm^2 and the estimated dose at 16 μm averaged over an area of 1 cm^2. The average doses over an area of 1 cm^2 are based on measurements using an extrapolation chamber electrode of area 1.1 mm^2 together with subsidiary data from Wells (1986, 1988) and Wells et al (1982) and subsidiary beta dosimetry calculations using the computer codes BETA (Bailey 1973) and VARSKIN (Traub et al 1987); see table 3. These ratios (table 3) have been used in figure 1 to indicate how the ED_{10} values (doses for a 10% incidence of the acute skin response) for pig skin (Hopewell 1989) averaged over 1 cm^2 at depths of 16 μm, depend upon the source size. Since the acute effect for large area sources arises due to the response of cells near the dermal/epidermal junction (and the suprabasal cells in the case of ^{147}Pm) the dose limit

should be related to the depth of these cells (in the vicinity of 100 μm). If a depth range of 100-150 μm is used then, for the larger sources, the ED_{10} values are reduced compared to the values at 16 μm by ≤ 2, ≤ 2 and ≤ 10 for $^{90}Sr/Y$, ^{170}Tm and ^{147} Pm respectively , and are all then in good agreement (see also figure 1; Table 3 and footnote c)

For small sources the ED_{10} doses (averaged over an area of 1 cm^2 at a depth of 16 μm, 1.6 mg cm^{-2} in tissue) are about 3 Gy (figure 3). Threshold doses are, for practical purposes, considered to be perhaps 2/3 of the ED_{10} values. For consistency with the procedure recommended for large area exposures it would appear to be appropriate to define a dose limit for small area exposures (including 'hot particles') at a depth in the vicinity of 100 μm. This choice of depth does however have an important impact on the appropriate value of a dose limit for poorly penetrating, low energy beta radiations for which the depth doses vary considerably over depths of even a few tens of μm. To date however there are no published data on the acute effects of radioactive particle exposures (source diameter < 1mm) for low energy beta emitters. When such data are available it may be necessary to revise the choice of depth to which the dose limit is related. With this reservation, and in view of philosophical as well as practical uncertainties involved in determining the threshold, a dose limit of 1 Gy (averaged over 1 cm^2 at a depth of 100-150 μm) appears to be satisfactory for the practical prevention of transient acute ulceration following 'hot particle' exposures. Although knowledge of hot particle effects has increased substantially, more information is required to relate the measured doses in these studies to other possible dose parameters and to provide a bridge between measured and calculated beta doses, particularly for small radioactive sources. Work is in progress to provide these data and to extend animal studies to source sizes below 1 mm for a range of beta radiation energies, including ^{60}Co (E_{max} 0.3 MeV).

3.2 Late effects.

Late dermal atrophy, which has been observed in pig and man following X-irradiation has also been observed following acute beta exposure studies for the two higher energy sources but not in the case of ^{147}Pm (Hamlet et al 1986). The dose-related changes in late skin effects indicates that dermal atrophy (visible as an induration) can occur at doses below the threshold for acute epidermal breakdown and may thus be a more limiting detrimental end-point following acute exposures. For similar magnitudes of slight dermal atrophy there is a factor of about 2-3 difference in the surface dose for $^{90}Sr/Y$ and ^{170}Tm which can be reconciled if a dose measurement is made in the mid to upper dermis at a depth in the region of 0.5 mm. For late telangiectasia in human skin the ED_{50} value for a moderate severity at 5 years was about 65 Gy for total doses given at 2 Gy per fraction (Tureson and Notter 1986). The threshold dose was about 40 Gy (equivalent single dose about 12.5 Gy). Clinically evident late atrophy in large fields given 30 fractions that were associated with a 50% incidence of a visible effect was about 69 Gy (Reinhold et al 1989) with a threshold dose of about 40 Gy and an equivalent single dose of about 10 Gy. Since these observed detrimental clinical effects are of dermal origin a dose measurement in the dermis rather than the epidermis would again appear to be appropriate.

4. Stochastic versus non-stochastic effects

It is necessary to bring together the discussions of stochastic and non-stochastic effects and to consider which of these effects is likely to be limiting in practice. A schematic amalgamation of the dependence of stochastic and non-stochastic dose limits on the area irradiated is indicated in figure 2. The non stochastic dose limits have been taken from radiotherapy data. The stochastic dose limit is based on an assumed upper limit of acceptable life-time mortality risk of $2x10^{-2}$. This was derived using a current ICRP risk figure for whole body exposure of $10^{-2}Sv^{-1}$ and an upper limit of life-time exposure of 50 mSv x 40 years. The skin cancer risk has been assumed to be proportional to the area of skin exposed. Skin cancer induction may be the limiting biological response for large area exposures, particularly if a mortality risk figure at the higher end of the indicated range is chosen by including a weighting W_D for non-fatal cancers in excess of 1%. If a relative risk model is used to predict life-time radiation induced skin cancer incidence, and if a detriment weighting factor W_D of 10% were to be applied, then an effective risk factor of 10^{-2} Sv^{-1} would apply for detrimental stochastic skin effects. An intermediate situation is the most plausible. In situations where cancer is considered limiting the relevant monitoring depth will be that of the epidermal basal layer and a conservative depth range would be between 20-100 μm (2-10 mg cm^2).

Depending upon the choice of the value of the stochastic risk figure, there is an area of exposed skin below which there is a transition to dose limitation on the basis of non-stochastic effects. For any reasonable value of stochastic risk it is apparent that for those relatively small body areas normally exposed to the highest skin doses, such as the fingers or hands, the limiting end point will be non-stochastic, and the relevant monitoring depth will be deeper, at some point in the dermis.

The risk of cancer from a 'hot particle' can be readily evaluated using the dose limit criteria of 1 Sv averaged over an area of 1 cm^2 and the skin cancer risk figures per cm^2 derived from Table 1 using a value for total skin area of about 2 x 10^4 cm^2. This leads to a mortality risk of 10^{-8} for a hot particle exposure in the region of the non stochastic effect threshold dose (using a relative risk model). The mortality risk for a single hot particle exposure therefore is negligible and limitation of such skin exposures should be on the basis of prevention of acute tissue breakdown.

5. Conclusions

On the basis of the data discussed here it is possible to suggest some guidance for the limitation of radiation exposure of the skin.

5.1 Whole body skin exposures

5.1.1 Non stochastic effects.

In order to prevent late detrimental cosmetic effects due to chronic dermal exposure a life time dose limit (based on clinical data and supported by animal studies) should be less than 40 Gy. The current ICRP

life-time dose limit of 20 Gy (0.5 Gy per year) if maintained, would represent a margin of safety. The dose should be evaluated in the upper dermis at a depth in the region of 500 μm. A suitable depth range to facilitate practical measurements would be between 300-500 μm (30-50 mg cm^{-2})

5.1.2 Stochastic Effects (skin cancer).

On the basis of a mortality risk figure of 2×10^{-4} Sv^{-1} and an upper limit of acceptable risk of 5×10^{-4} per year, an occupational life time dose limit would be 100 Sv (2.5 Sv per year). The dose should be evaluated to the basal cells of the epidermis which can conservatively be taken to be at a depth between 20 -100 μm (2-10 mg cm^{-2}).

5.2 Partial body skin exposures

5.2.1. Non stochastic effects.

The discussion of a non-stochastic dose limit, given in 5.1.1 should be considered to be independent of the area exposed, subject to comments in 5.3.

5.2.2. Stochastic effects (skin cancer).

The discussion of a dose limit for stochastic effects given in 5.1.2 should also apply to exposures involving all of the areas of the body normally subject to UV exposure (face hands and arms) since the skin cancer risk figure, on which the dose limit is based, is dominated by excess skin cancers reported in these skin areas. For smaller areas of skin the dose limit, on the basis of skin cancer mortality, will be proportionally higher.

5.3 'Hot particle' exposures.

'Hot particle' exposures are spatially non-uniform exposures from discrete radioactive sources with dimensions of the order of less than or about 1 mm. Highly localised re-irradiation of the same skin site is normally considered to be highly unlikely. Early acute ulceration is considered to be the end-point of concern because of the possibility of infection. Early acute ulceration should be prevented if the average dose over an area of 1 cm^2, measured between depths of 100-150 μm (10-15 mg cm^{-2}) delivered within a few hours, is restricted to 1 Sv. Alternative exposure limitation criteria, such as total number of beta particles emitted, could be used. Revision of this recommendation should be considered when further data on 'hot particle' skin exposures for low energy beta emitters become available.

5.4 General comments on skin dosemeter design.

In order to comply with both the stochastic and non-stochastic dose limits, for routine chronic exposures, personal skin dosemeters may need to provide measurements of dose at 2 depths relevant to dermal (300-500 μm) and epidermal (20-100 μm) biological effects. An alternative conservative procedure could involve only one measurement at the

superficial depth (20-100 µm) with the application of the lower dose limit relevant to the dermal response (say 0.5 Sv per year). Such a procedure would be essentially the same as that currently adopted in several countries using the recently available thin dosemeters designed to measure dose between 50 and 100 µm in accord with ICRP publication 26. In situations where depth dose information is available it may be possible to justify the evaluation of the dermal dose alone using an appropriate dosemeter with a thicker and deeper detector element, such as one of the many thermoluminescence dosemeter (TLD) designs which have been available for some years

If a skin dosemeter is required to provide clinical prognostic information in the event of an accidental over-exposure then the use of a device with at least 2 detection elements will be required.

The thickness of a routine personal skin dosemeter is clearly of importance in providing the necessary energy response of the detector. The area of the dosemeter, for exposures other than those due to 'hot particles', is not a particularly important parameter from a biological standpoint but may be relevant to questions related to the radiation sensitivity of the device. Most currently used TLD dosemeters, such as those of the sachet type with areas of about 1 cm^2, for example, would be appropriate and could also be used to provide 'hot particle' dose assessments using the criterion of average dose.

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For further references see section V.

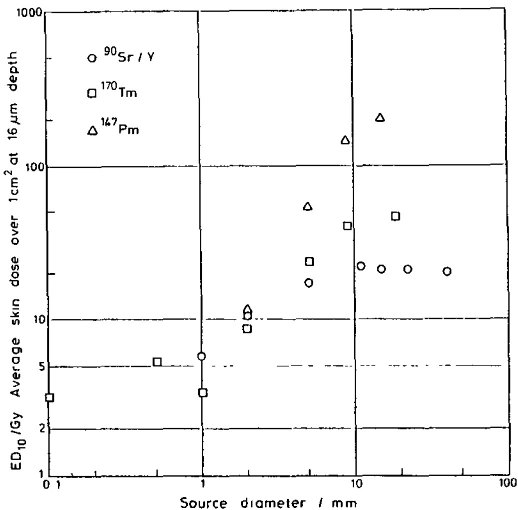

Figure 1. The ED_{10} value for acute tissue breakdown in pig skin, averaged over an area of 1 cm^2 at a depth of 16 μm, is indicated as a function of the diameter of the radiation source. Values are based on the data of tables 2 and 3 using extrapolation chamber measurements and calculations to relate the dose averaged over 1 cm^2 to the directly measured dose averaged over 1.1 mm^2.

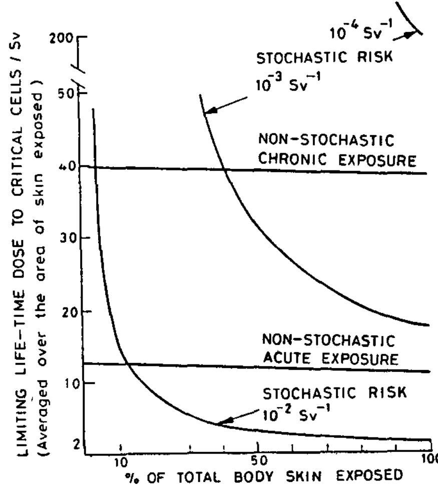

Figure 2. Schematic dependence of stochastic and non-stochastic dose limits on area of irradiated skin. Non-stochastic dose limits are based on radiotherapy data. The stochastic dose limit is based on the assumption of an acceptable life-time mortality risk of 2 x 10^{-2} (see text). The indicated stochastic risk figures are for life-time mortality.

TABLE 2.

ACUTE BREAKDOWN OF PIG SKIN FOLLOWING BETA RADIATION EXPOSURE.

VALUES OF ED_{50} AND ED_{10}
DOSES AVERAGED OVER AN AREA OF 1.1 mm^2 AT A DEPTH OF 16 µm

ISOTOPE	SOURCE DIAMETER /mm	ED_{50} (a) /Gy	ED_{10} (a) /Gy	TYPE OF LESION
^{90}Sr/Y	40	28.6±1.4	20.1±1.4	MD
	22.5	27.3±0.7	21.1±1.2	MD
	15	42.3±4.8	21.0±3.4	MD
	11	44.8±4.5	22.0±3.2	MD
	5	70.3±5.0	34.4±4.0	MD
	2	119 ±9	82 ±10	AU
	1	253 ±31	104 ±25	AU
^{170}Tm	19	71.9±5.7	47.5±4.2	MD
	9	73.9±2.6	48.8±2.8	MD
	5	85.8±4.6	56.7±3.9	MD
	2	179 ±15	77.7±9.4	AU
	1	202 ±24	87.7±12.6	AU
	0.5	339 ±36	147 ±20	AU
	0.1	197 ±25	85.7±13.1	AU
^{147}Pm (b)	1	341 ±22	203 ±27	AEN
	9	381 ±23	244 ±30	AEN
	5	410 ±22	272 ±27	AEN
	2	478 ±26	340 ±27	AEN

(a) Measured dose at a depth of 16µm, averaged over an electrode area of 1.1 mm^2
(b) Surface doses for ^{147}Pm are about 50% higher than at 16 µm.

MD - moist desquamation
AU - acute ulceration
AEN - acute epidermal necrosis

TABLE 3

ACUTE BREAKDOWN OF PIG SKIN FOLLOWING BETA RADIATION EXPOSURE.

THE RATIO BETWEEN
MEASURED DOSES[a] AND DOSES AVERAGED OVER AN AREA OF 1 cm^2

ISOTOPE	SOURCE DIAMETER /mm	[a]AVERAGE DOSE OVER 1.1 mm^2 AT 16 µm / AVERAGE DOSE OVER $1cm^2$		
		RATIO BASED ON [b]measurement of average dose over 1 cm^2	RATIO BASED ON [c]calculation of average dose over 1 cm^2	[d]RATIO USED IN FIGURE 1
^{90}Sr/Y	40	1	1	1
	22.5	1	1	1
	15	-	1.03	1
	11	-	1.1	1
	5	-	5	2
	2	-	28	7
	1	18	69	18
^{170}Tm	19	1	1	1
	9	-	1.6	1.2
	5	-	5	2.4
	2	8.7	28.7	8.7
	1	25	70	25
	0.5	-	75	27
	0.1	-	75	27
^{147}Pm	15	1	1	1
	9	1.6	1.6	1.6
	5	5	5	5
	2	32	32	32

(a) The majority of dose measurements were made using an extrapolation chamber with an electrode of area 1.1 mm^2 at a depth of 16 µm.

(b) This ratio is based on extrapolation chamber measurements at an equivalent tissue depth of 16 µm, using several different sized electrodes (Wells et al ,1982; Wells, 1988).

(c) The ratio is based on a simplified calculation of doses at a depth of 16 µm, using a plane source geometry, neglecting self absorption (Bailey, 1973; Traub et al, 1987). Based on such calculations the ratio is increased by factors of about ≤ 2, ≤ 2 and ≤ 10 for ^{90}Sr/Y, ^{170}Tm and ^{147}Pm respectively, when the average dose over an area of $1cm^2$ is calculated at a depth of 100-150 µm.

(d) Measured values are used where available. The ratio of calculated values for various source sizes has been used to guide interpolation of measured values.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

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V. Publications:

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-C-062-UK

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Title of the research contract:

Cellular analysis and dose-response relationships in long-term radiation injury to mouse bone marrow.

List of projects:

1. Determination of long-term injury of radiation to mouse bone marrow.

Title of the project no.:

Determination of long-term injury of radiation to mouse bone marrow

Head(s) of project:

J.H. Hendry

Scientific staff:

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I. Objectives of the project:

To investigate residual haemopoietic injury in terms of deficiencies in various progenitor and maturing cell populations, after irradiation delivered acutely, at low dose rate, or using various fractionation regimens.

II. Objectives for the reporting period:

1) To study acute responses of haemopoietic progenitor cells (e.g. CFU-S, GM-CFC) and stromal progenitor cells (CFU-F, ossicle-forming cells), variously in murine, canine and human marrow.

2) To assess recovery and residual injury in the various cell populations, using also long-term marrow cultures, and to interpret dose-response relationships in terms of deficiencies in particular subpopulations of cells and in the interactions between them.

III. Progress achieved:

1. METHODOLOGY

1.1 Bone marrow sources Murine, canine, and human marrow were used variously in these studies. Femoral marrow was used from 10-12 week old $B6D2F_1$ mice. Cell suspensions were made using Fischer's or Iscove's medium.

A semi-purified population of primitive cells (FACS-BM) was obtained by using a metrizamide density cut (1.089 ml^{-1}) followed by sorting twice on a FACS machine. The population collected contained 95% CFU-S and MIX-CFC (a 20-fold enrichment). Bi-potential CFC were obtained from marrow 3 days after injection of mice with 200 mg kg^{-1} CP. The marrow cells were subjected to a metrizamide density cut, followed by collection of fractions at 24-36 ml min^{-1} using a counterflow centrifugal elutriator. CCE-BM was highly enriched for CCM-CFC.

Rib marrow was used from adult Beagle dogs. Sternal or iliac crest marrow was used from haematologically normal male and female patients undergoing diagnostic procedures, or from rib segments removed for access during surgery. Canine and human marrow samples were layered on to an equal volume of lymphocyte separation medium (density 1.077 g ml^{-1}, Flow Labs) at room temperature. The cells were centrifuged, and the interface aspirated and washed twice with Iscove's-Modified-Dulbecco's medium.

1.2 Irradiations These were variously (a) ^{60}Co gamma-rays at dose-rates between 0.0005 and 0.05 Gy min^{-1}, (b) ^{137}Cs gamma-rays at 4 Gy min^{-1}, and (c) 300 kVp X-rays (10 mA, hvl=2.3 mm Cu) at 0.6-0.7 Gy min^{-1}.

1.3 CFU-S Recipient mice received 15.5 Gy ^{60}Co gamma-rays at 0.016 Gy min^{-1}. Colonies were counted at day 9 and 11. CFU-S per 11-day colony were assessed by secondary transplantation. CFU-S in axial and marginal femoral-marrow samples were removed using different sized syringe needles. Progenitor cell cycling was measured using thymidine suicide (7.4 MBq ^{3}HTdR in 0.2 ml medium, specific activity 555 GBq $mmole^{-1}$). CFU-S stimulator was prepared as conditioned medium after short or long incubation times. Stimulating activity was assessed using 3h or 18h incubations with 'responder' cells followed by thymidine suicide measurements.

1.4 In vitro CFC For mouse marrow, Iscove's medium was used with 20% horse serum, 0.3% agar and one or more of the following factors at optimal concentrations: IL-1, recombinant IL-3 (a gift from Amgen), recombinant human (rh) GM-CSF (a gift from Biogen), partially purified murine M-CSF, (rh) G-CSF (Amgen). For canine marrow, 20% foetal calf serum or 10% irradiated-dog serum (a gift from Dr. W. Nothdurft, Ulm University) was used together with 1% bovine serum albumin, and one or more of the above factors or 10^6 WBC ml^{-1}, 15% PHA-conditioned medium, or 20% 5637-CM. For human marrow, 20% foetal calf serum was used together with the above purified or recombinant growth factors. Cultures were incubated in humidified 5% Co_2, 5% O_2 and 90% N_2 at 37°C for 7-12 days.

1.5 CFU-F Cultures were set up using Iscove's medium, and 5% foetal calf serum. They were incubated in 5% CO_2, 95% N_2 at 37°C for 7 days.

1.6 Ossicles A plug of femoral marrow was inserted under the kidney capsule of recipient mice, and the ossicle produced was assayed for CFU-S after 8 weeks.

1.7 Long-term cultures Cultures were established using 1 femur in 10 ml Fischer's medium supplemented with 20% pretested horse serum and 10^{-6} M hydrocortisone. The cultures were fed weekly by demipopulation. Total sulphated GAGS were detected by co-precipitation using chondroitin sulphate as carrier in an excess of CPC.

2. RESULTS AND DISCUSSION

2.1 Acute Responses

2.1.1 Haemopoietic cells

Cultures of γ-irradiated murine bone-marrow cells were grown using different colony-stimulating factors (CSF) to analyse in detail the radiosensitivity of the colony-forming cells (CFC) in the haemopoietic hierarchy (Baird 1988). With unfractionated bone-marrow the Do values ranged from 1.3 Gy (GM-CSF) to 1.0 Gy (M-CSF), all with n = 1. However, when IL-3 was used the Do was 1.2 Gy and the survival curve had a shoulder (n = 1.9). A combination of IL-3 with IL-1 resulted in a Do of 1.2 Gy with n = 1, hence the IL-1 may somehow reduce the repair capacity of the CFC responsive to IL-3. When the cells were co-treated with M-CSF and IL-1 the Do was again 1.2 Gy with n = 1, indicating that the population stimulated may be the same population recruited by IL-3 with IL-1. The combination of IL-3 with M-CSF and with IL-1 produced a biphasic survival curve with a Do of 1.7 Gy for the resistant population (60%), suggesting that these CSF-s may have stimulated a sub-population of resistant progenitors. These data indicate that the use of different CSF-s can influence the shape of the survival curve for GM-CFC and that the progenitor cells become increasingly more radiosensitive as they progress through development.

With semi-purified populations of primitive (FACS-BM) or bipotential CFC (CCE-BM), Do values were generally lower with respect to the equivalent values for unpurified bone marrow (range 62 ± 7 cGy to 135 ± cGy). This suggested that factors produced by stimulated accessory cells may influence the subpopulation selection and hence the measured radiosensitivity of the CFC.

Cultures of γ-irradiated canine bone marrow cells were grown for 10 days using different colony-stimulating factors (CSF) at optimal concentrations. These included 10% serum (DS) from dogs irradiated 9 days previously with 3.9 Gy (kindly provided by Dr W. Nothdurft, Ulm University) together with 10^6 white blood cells (WBC) per ml, 15% PHA-conditioned medium (CM), 20% 5637-CM, 3000 U/ml human rGM-CSF, and 5000 U/ml human rG-CSF. Sensitivity parameters are shown in Table 1. There was no significant shoulder on the survival curve using DS + WBC or 5637-CM, but there was with PHA-CM, measured as the mean of values in several experiments. The curves determined using rGM-CSF and rG-CSF showed significant shoulders but the latter was assessed in only one experiment. Average values of colony-forming efficiency (CFE) were similar when using different factors, and Do values were about 50 cGy (up to 70 cGy) or less. There was no clear relationship between CFE and Do over an extreme range of about 7 in values of CFE. Cluster (2 to 49 cells) : colony (≥ 50 cells) ratios increased from about 1 in controls to about 5 after 1 Gy. These studies confirm other data in the literature using irradiated dog serum as a source of stimulator which show that progenitor cells in the dog are more radiosensitive than in mouse or in man (see below).

Table 1. Radiosensitivity of haemopoietic progenitor cells

	CSF	CFE %	Do ± sem (cGy)	n
Dog	DS + WBC[b]	0.05	50 ± 5	1.9 ± 0.4[a]
			(71 ± 7	1.0)
	PHA-CM	0.08	41 ± 4	2.0 ± 0.3
			(64 ± 3	1.0)
	5637-CM	0.06	40 ± 5	1.5 ± 0.3[a]
			(50 ± 2	1.0)
	rhGM	0.05	33 ± 6	2.2 ± 0.7
			(52 ± 4	1.0)
	rhG[b]	0.06	21 ± 5	6.2 ± 3.8
			(52 ± 11	1.0)
Man	h5637-CM	0.09	138 ± 12	1.0 ± 0.1[a]
			(130 ± 4	1.0)
	rhIL-3	0.14	124 ± 16	1.0 ± 0.1[a]
			(122 ± 6	1.0)
	rhGM[b]	0.14	115 ± 15	1.4 ± 0.2[a]
			(138 ± 7	1.0)
	rhG[b]	0.08	100 ± 21	1.0 ± 0.3[a]
			(100 ± 8	1.0)

[a] = not significantly different from 1.0
[b] = 1 experiment only

Human haemopoietic cells were obtained from rib samples or aspirates (Baird _et al_ 1989). Optimal concentrations of the human CSF were lower for human CFC than for dog CFC, being 1000 U/ml rhGM-CSF or G-CSF, 500 ng/ml rhIL-3, and 10% h5637-CM. The colonies were counted between days 9 and 12 of growth. All the survival curves were exponential, and there was no significant correlations between radiosensitivity and colony-forming efficiency (CFE) among different experiments. Mean values of the survival parameters among several experiments are quoted in Table 1, although there was a significant difference between experiments in the case of IL-3 and G-CSF. The use of rhG-CSF gave the lowest Do value of 100 cGy.

A comparison of these data for dog and man with those for mouse shows the following features:

(a) The human CSF had a higher activity on human than on dog CFC,

(b) A higher degree of biological variation was evident in human samples than in mouse or dog samples,

(c) Different proportions of CFC are stimulated by the same CSF in different species, and some take longer to produce colonies,

(d) The CFC stimulated in dog bone marrow are more radiosensitive compared to human and mouse, and the latter show some similarities in radiosensitivity,

(e) G-CSF recruits the most radiosensitive subpopulation in all 3 species, and in general radiosensitivity increases with differentiation status.

(f) Among the different experiments with a given CSF, the radiosensitivity was not related to the CFE,

(g) The increase in cluster:colony ratio with increasing dose was more marked in the case of the dog than the mouse, and this may reflect or be the cause of the higher apparent sensitivity of haemopoietic cells in the dog.

The analysis of 68 published sets of dose-incidence data (Hendry and Roberts, 1989) for marrow failure in different species, using a double-log mortality function, has indicated:

1) There is more heterogeneity, i.e. greater sums-of-squares per degree of freedom, within the data sets for mouse than for larger species (monkey, dog, sheep, goat, pig).

2) For mice the curves for acute doses are characterized by a D_o of about 100 cGy for tissue-rescuing units (or target cells), which are depleted at most to about 3 x 10^{-4} at LD_{50}.
3) Larger species are much less tolerant to target-cell depletion, the corresponding level being consistently in the range of 10^{-2} - 10^{-3} at LD_{50}. Also, the D_o is often lower (∿55cGy), which is compatible in the dog with such a value for hemopoietic progenitor cells.
4) With larger species there is an unexpected reduction in heterogeneity when the dose rate is lower, which gives a D_o lower than expected and a higher extrapolate.

It is concluded that the position and slope of the dose-incidence curves are compatible with interpretations based primarily on target-cell number and survival characteristics, modified by additional heterogeneity factors.

2.1.2 Stromal cells

Mice were irradiated to the whole-body with cobalt-60 gamma-rays at a dose-rate of 0.7 Gy per day for 30 days (Wang et al, 1987). The number of fibroblastic colony forming units (CFU-F) was assessed in femoral marrow taken from mice killed at various days during and after irradiation. Colonies comprising mainly fibroblasts but also endothelial cells and macrophages were grown in agar and counted at day 7 of growth. The number of CFU-F declined at a rate which was nearly exponential, and there was little evidence for a threshold. The decline was characterised by a Do value of about 2.5 Gy down to a surviving fraction of 0.1 (total dose about 7 Gy). After higher doses the resolution of the assay decreased, but the exponential decline appeared to continue with a somewhat lesser slope. After an accumulated dose of 9 Gy, recovery was seen after a lag period of about 10 days. The recovery reached a plateau at about 40% of control by 25 days, and this was followed by a further recovery phase between 2 and 5 months taking the CFU-F content back to near normal. After 20 Gy, the lag period before recovery was less, and the plateau was reached early at about 18 days. The later period of recovery was absent. The rate of decline in CFU-F content during irradiation was similar to that found previously using higher dose-rates of 0.045 or 0.014 Gy per minute, and this suggests that the limiting initial slope of the survival curve was reached using all these dose-rates. Also, the sensitivity was less than

that found for haemopoietic stem cells (CFU-S) by a factor of about 2.5. The near-plateau in CFU-S at about 1 % of control during extended continuous irradiation was not seen clearly for the CFU-F, suggesting that there was no increased cycling and proliferation of CFU-F during irradiation. Recovery of the CFU-F population after 9 Gy accumulated doses was slower than for CFU-S, but after 20 Gy the levels of CFU-F and CFU-S were within a factor of 2 during the recovery period.

The kidney implant was made from the whole femoral marrow plug taken from control or irradiated mice and transplanted into untreated recipient mice either immediately or at 8 to 22 weeks after irradiation of the donor. The CFU-S content of the ossicles declined approximately exponentially with increasing dose at 4.2 Gy/minute, and the D_o value was about 1.5 Gy (Molineux et al, 1987). This was similar to that found for CFU-F in the irradiated femora. There was a sparing effect of the low dose-rate of 0.016 Gy per minute, particularly after high doses. After 10 Gy delivered at high dose-rate, no ossicles formed, in contrast to after the low dose-rate where ossicles were formed containing 10% of the number of CFU-S in control ossicles.

The similarity of the sensitivity of CFU-F and the cells forming the ossicle is expected if the CFU-F are the primary progenitor cells of the ossicle, possibly requiring secondary associated (radioresistant) cell populations for development. Other studies have suggested that the CFU-F form an important part of the haemopoietic microenvironment, recently for example by the similarities between cells in colonies derived from CFU-F and in the stromal component of long-term cultures. Also of note is the marked sparing of low dose-rate irradiation. On average over the dose range used of 0-10 Gy the sparing was similar in magnitude for CFU-F and for the ossicle-forming cells, although for the latter the curve tended to be biphasic with more sparing than for CFU-F after high doses (7.5 and 10 Gy) than for low doses (2.5 and 5 Gy).

2.2 Late responses

2.2.1 Progenitor cells

Following irradiation given at 4.2 Gy per minute, recovery of CFU-S in the femur had reached 70% by 3 weeks after doses up to 4.5 Gy, and 40% after 6.5 Gy (Gallini et al, 1988). These values increased to 100% and

70% by 3 months. GM-CFC showed less recovery at 3 weeks, but showed an overshoot at 3 months. Thereafter, both CFU-S and GM-CFC numbers declined to reach 30-50% of control at 9 months after 6.5 Gy, with higher levels after lower doses. The numbers of CFU-F recovered much more slowly, and at 6 months after 6.5 Gy they had reached only 20% of control (again with higher levels after lower doses).

After irradiation at 0.0005 Gy/minute, there was little change in the CFU-S content of the femur between 3 weeks and 9 months, when recovery had reached about 70% after all doses between 1.5 and 12.5 Gy. GM-CFC were also at about 70% of control at 9 months. The long-term levels of CFU-F were higher at all times of assay from 3 weeks after high doses given using the low instead of the high dose-rate, reaching 40-70% of control at 9 months after doses from 1.5-12.5 Gy.

Long-term recovery of CFU-S and GM-CFC in the femora of irradiated mice was incomplete up to 9 months after doses up to 6.5 Gy given at either high or low dose-rate. After 6.5 Gy at high dose-rates, the levels of CFU-F at 6 months were lower than those for the haemopoietic progenitors, but this difference was less marked when low dose-rate was used. This indicates that the greater recovered levels of CFU-F following low dose-rate irradiation did not help restore the full complement of haemopoietic progenitors. Either there is no apparent need for full regeneration - increased cycling and/or amplification coping adequately - or the micro-environment is more suboptimal in the long-term than indicated solely by CFU-F numbers.

The correlation between the acute response of CFU-F and the ossicle-forming cells was not borne out by the subsequent recovery in the irradiated donor mouse. When a delay of 8 or 22 weeks was introduced between the irradiation and the removal of the marrow plug for implantation, there was recovery by a maximum factor of 2-3 in terms of numbers of CFU-S in the ossicles, after both dose-rates and at both times of assay (Molineux et al, 1988). The recovery was much greater for numbers of CFU-F in the femur, being a factor of 10-20 after 7.5-10Gy. This suggests that CFU-F may be only one component of the stromal elements required for ossicle formation, with other components developing injury slowly and requiring longer times for recovery.

Groups of mice were given 15 fractions (5 per week) whole-body gamma-irradiation (0.064 Gy per minute) using equal doses per fraction, and the dose per fraction ranged from 0.7 down to 0.1 Gy per fraction. Other groups received one course of 15 fractions, followed after a recovery interval of 3 weeks, by a second course of 15 fractions (Gallini et al, 1989). At day 3 after the last of 15 fractions, there was a dose-dependent reduction in the number of CFU-S per femur, characterised by a Do value of about 3 Gy. There was marked recovery by 3 weeks to about 50% of the age-matched controls, with little dose-dependence being demonstrated. There was little further recovery, and by 9-12 months numbers of CFU-S were still subnormal (60-80%) even after the smallest total doses used of 1.5 Gy.

GM-CFC were less affected than CFU-S at day 3 after the last dose. Recovery improved to 6 months and then later declined, and by 12 months the levels of CFU-S and GM-CFC were similar. Values of CFU-S per colony were reduced at 3 days and at 3 weeks after the last dose. Later there was recovery but by 6-12 months after the highest total dose of 10.5 Gy the values were about half normal. CFU-F were less affected than the CFU-S and GM-CFC at day 3 after the last dose, and there was good recovery in the longterm after the lower doses. The data for CFU-S/ossicle were more scattered, but overall they showed a similar pattern to that seen for CFU-F. In the long-term the levels of CFU-S after the higher accumulated doses were slightly lower suggesting greater injury by higher accumulated doses. Stem-cell quality, assessed by CFU-S per colony, showed a consistent reduction after the highest accumulated dose of 21 Gy delivered in 30 fractions.

The rate of cycling of stem cells (CFU-S) and granulocyte-macrophage colony-forming cells (GM-CFC) was measured in the femoral marrow of mice at various times after whole-body irradiation with 1.5-10 Gy or 4 doses of 4.5 Gy (each separated by 3 weeks) (Tejero et al, 1988). A dose-dependent increase in the cycling rate of both CFU-S and GM-CFC was observed which was similar at all times of assay between 3 weeks and 36 months. This invariance with time was in contrast to in controls, where for GM-CFC the cycling rate declined unexpectedly with increasing age of the mice. This latter observation is of importance in the use of cycle-specific cytotoxic drugs where less effect on this important cell

population may be found in older animals, and possibly in man. The cycling of CFU-S and GM-CFC was also increased at 6-10 months after 4 doses of 4.5 Gy, and the femoral content of these precursor cells was reduced more than after single doses. In addition, when assessing separately the central core of marrow and the peripheral cylinder inside the bone shaft, it was found that the concentration of CFU-S was similar in both regions. This is in contrast with the higher concentration found in marginal than in axial marrow in controls. Assessment of the level and production of a stimulator of CFU-S cycling showed that the level was elevated in both axial and marginal marrow fractions, but the rate of production of stimulator was much reduced.

The acute recovery of populations of day 11 CFU-S, iv-CFC and CFU-F in mouse bone marrow, following a test dose of X-rays, cyclophosphamide (CP) or busulphan given to mice previously treated with repeated priming doses, was in general predictable from the amount of residual injury after the priming doses. A marked exception was iv-CFC after X-rays, which although amplified to near normal levels during the residual injury phase, recovered after the test irradiation from low levels of CFU-S. The amount of residual injury after sequential treatments of different agents was in general less than expected on the basis of the product of the effects of the individual agents. This was most marked for CP priming treatments, where the long-term recovery of day 11 CFU-S after the test dose remained persistently above control levels. Also, some correlation was found between improved stromal recovery (CFU-F) and the CFU-S content following the sequential treatment protocols.

2.2.2 Long-term cultures

Injury inflicted upon the marrow stroma following whole-body irradiation and its repair over a one year period has been assessed in primary long-term bone marrow cultures established at increasing time intervals post irradiation (Bierkens et al, 1989). Different doses (4.5, 10, 4x4.5 Gy) at different dose-rates (1.6 cGy/min and 0.05 cGy/min) were chosen so as to maximise the sparing effect in the stroma. The stromal cellularity in long-term cultures established shortly after irradiation was reduced by 40 to 90% depending on the applied dose and dose-rate. As a consequence of the poor repopulation ability of the stroma the cumulative CFU-S and GM-CFC production dropped sharply by 70 to 100% of

the levels in control cultures. The sparing which occurred in the stroma at low dose-rate was reflected at the hemopoietic precursor cell level as a higher incidence of CFU-S and GM-CFC progenitors. A slow recovery of the stroma to generate an adherent layer was paralleled by increasing numbers of CFU-S and GM-CFC. Whereas the stromal regeneration was near complete 1 year after irradiation, the CFU-S and GM-CFC levels reached only between 50 and 80% of the levels in control cultures. Cultures established from 4x4.5 Gy mice performed poorly over the entire 1 year period. Sulphated GAGs which have been implicated in the establishment of the functional integrity of the stroma, did not constitute an aspect of the radiation damage to the marrow stroma at any time after irradiation.

A parallel set of experiments was performed to assess the ability of stromal layers established from irradiated mice over a one year period (as above) to 'home' a fresh innoculum of haemopoietic cells. The resumption of ability of the stroma to induce active haemopoiesis was shown to be dependent on its ability to generate a fully established layer. As for primary cultures, one year after irradiation the stromal layers had regained their ability to support prolonged proliferation and differentiation of a fresh marrow innoculum. Both these results suggest that the proliferative regeneration of the stroma is paralleled by the resumption of its ability to support active hemopoiesis. No evidence was provided for persistent defects in the functioning of the stroma following doses up to 10 Gy to the whole body at low dose-rate. In contrast, non-adherent cells produced in primary cultures established at increasing intervals after irradiation performed poorly when reinoculated on 'normal' stromal layers. Their ability to repopulate a stromal environment was dependent on both the dose and the dose-rate initially delivered to the donor mice.

3. CONCLUSIONS

These studies have greatly extended our knowledge of the radiation response of the haemopoietic system in different species, in particular concerning long-term responses and the effects of changing dose-rate or fractionation patterns. A correlation is drawn between haemopoietic and stromal recovery, the latter being responsible for slow but extensive haemopoietic recovery after very low dose-rate irradiation.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

C. Tejero, Dept. of Bioquimica, Universidad Complutense de Madrid, Facultad de Veterinaria, Cuidad University, 28040 Madrid, Spain, Dr. W. Nothdurft, University of Ulm, Germany (supply of serum from irradiated dogs), and other EULEP groups for discussions.

V. Publications

MOLINEUX, G., TESTA, N.G., HENDRY, J.H. & SCHOFIELD, R. (1987). The radiation sensitivity of the haemopoietic microenvironment - effect of dose rate on ectopic ossicle formation. Radiotherapy and Oncology, 10: 157-161.

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GALLINI, R., HENDRY, J.H., MOLINEUX, G. & TESTA, N.G. (1988). The effect of low dose rate on recovery of hemopoietic and stromal progenitor cells in γ-irradiated mouse bone marrow. Radiation Research 115: 481-487.

TESTA, N.G., HENDRY, J.H. & MOLINEUX (1988). Long-term bone marrow damage after cytotoxic treatment: stem cells and microenvironment. In: Hematopoiesis, long-term effects of chemotherapy and radiation. Hematology 8: 75-81. Eds: NG Testa, RP Gale. Marcell Dekker, New York and Basel.

TEJERO, C., LORD, B.I., MASON, T.M., & HENDRY, J.H. (1988a). Long-term haemopoietic injury in mice after repeated irradiation: Precursor-cell cycling and its regulation. Eur. J. Haematol. 41: 278-284.

TEJERO, C., HENDRY, J.H. & TESTA, N.G. (1988b). Persistent dose-dependent increases in cycling of haemopoietic precursor cells after irradiation. Cell Tissue Kinet. 21: 201-204.

BAIRD, M.C. (1989). The radiosensitivity of haemopoietic cells in different species. Ph.D. thesis. University of Manchester, U.K.

TEJERO, C., TESTA, N.G. & HENDRY, J.H. (1989). Decline in cycling of granulocyte-macrophage colony-forming cells with increasing age in mice. Exp. Hematol. 17: 66-67.

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BIERKENS, J.G., HENDRY, J.H. & TESTA, N.G. (1989). The radiation response and recovery of bone marrow stroma with particular reference to long-term bone marrow cultures. Eur. J. Haematol. 43: 95-107.

HENDRY, J.H. & ROBERTS, S.A. Analysis of dose-incidence relationships for marrow failure in different species in terms of radio-sensitivity of tissue-rescuing units. Radiation Research (in press).

RADIATION PROTECTION PROGRAMME
Final Report

Contractor:

Contract no.: BI6-C-063-UK

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Title of the research contract:

Early and late effects of radiation on the skin.

List of projects:

1. Pathogenesis of early and late radiation damage to skin.
2. The biological effects of non-uniform irradiation on pig skin.

Title of the project no.: 1

Pathogenesis of early and late radiation damage to skin

Head(s) of project:

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Scientific staff:

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I. Objectives of the project:

The accidental exposure of the skin to ionising radiation can result in the development of a spectrum of early and late non stochastic changes. At present, there is confusion and a lack of understanding of the pathogenesis of these various effects and although a complex nomenclature has evolved to describe them, in some instances this has proved to be erroneous and/or misleading. A clear understanding of the effects of radiation on the skin and knowledge of the underlying pathogenesis of lesions, with the identification of target cells, is essential if accidental overexposures are to be treated successfully or effects due to occupational exposure are to be avoided by the setting of realistic dose-limits.

II. Objectives for the reporting period:

The pathogenesis of early and late radiation-induced changes to the skin have been evaluated extensively in the pig, since the skin of this species is both grossly and histologically similar to that of man. Early effects have been examined after irradiation with X-rays and β-rays from sources emitting a range of β-energies. The effects of changing source size has also been evaluated. Late radiation effects, represented largely by tissue atrophy, have been examined for periods up to two years after irradiation.

III. Progress achieved:

The results of extensive studies carried out on pig skin, over the period covered by the present research contract, have indicated that the type of radiation responses observed depend both on the area of skin exposed and on the energy of the radiation. Exposures resulting from contact with discreet radioactive particles ('hot' particles) or radioactive source of low energy, which are poorly penetrated into tissue, produce effects that are different mechanistically from those seen after more generalised exposure. This has not been recognised until recently and the usage of the same terminology to describe both sets of lesions has, in the past, caused considerable confusion.

EFFECTS OF GENERALISED EXPOSURE

Following more generalised exposure of the skin to ionising radiation several distinct phases of response have been identified and the possible underlying pathogenesis investigated. These phases of response include:-

a) a very early transient erythema
b) the main erythematous reaction, reflecting indirectly a varying severity of loss of basal cells; either a dry or a moist desquamatory response may be seen after 3-6 weeks, depending on the exposure dose
c) a late phase of erythema associated with dermal ischaemia and the possible development of necrosis after 8-16 weeks
d) the appearance of late skin atrophy after >26 weeks.

a) Early transient erythema

This was seen within a few hours of irradiation with doses ≧15Gy. It subsided after 24-48 hours. The underlying mechanisms responsible for this effect have not been investigated.

b) The main erythematous reaction

The results of investigations indicate that the target cell population, damage to which cause this response, is the basal cell population of the epidermis. The reddening of the skin is thought to represent a secondary inflammatory reaction. Within a few days of irradiation with X-rays a marked fall was found in the ^{3}H-thymidine labelling index of basal cells (Fig. 1). The mitotic index was also decreased and the number of degenerating cells slightly increased after single doses of 15 and 20Gy.

However, the process of cell death at mitosis may not be the major contributory factor, for if basal cells that are not reproductively viable simply differentiate and migrate in the usual way this would, even in the absence of any cell division, lead to the denudation the epidermis within a time scale approximately equivalent to that of its normal turnover. The loss of cells from the basal layer was found to occur at a rate of ~2.6%/day after single dose of 15 or 20Gy of X-rays (Fig. 2). This was close to the natural turnover rate in the epidermis. This rate of cell loss was independent of the radiation dose and would suggest, after higher doses, that total denudation of the epidermis would occur after a latent interval of 30-35 days. The time of appearance of spotting moist desquamation was 33.2 ± 1.1 days after single doses of >25Gy.

The repopulation of the basal layer of the epidermis after irradiation, with doses just above or just below the threshold for moist desquamation, is predominantly due to the proliferation of surviving clonogenic cells from within the irradiated area, however, with increasingly smaller areas of skin the migration of cells from the field edge may make a larger contribution. Such clusters of viable cells (colonies) are first seen just prior to the appearance of the peak skin reaction and can be identified as groups of cells with a near normal density (Fig. 2) and with a ^{3}H-thymidine labelling index as high as 40-50% (Fig. 1). A high proportion of these groups of labelled cells were associated with the canal of hair follicles.

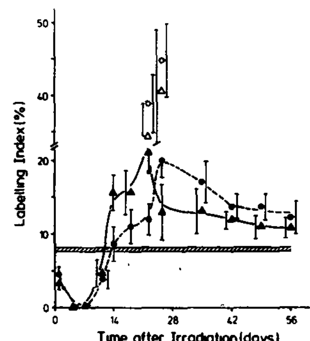

Figure 1: Time-course of the changes in the ^{3}H-thymidine labelling index (±SE) of basal cells in the epidermis of Large White pigs after irradiation with single doses of 15Gy (▲) and 20Gy (●) of X-rays. The open symbols indiated the labelling index in the cell colonies and the hatched area the value (± SE) in unirradiated skin.

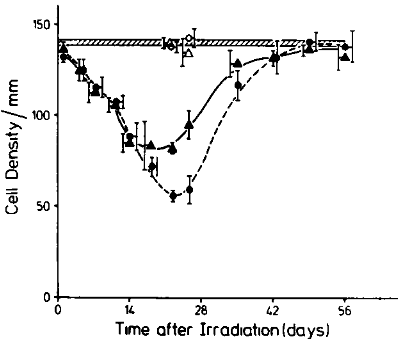

Figure 2: Time-course of the changes in the density of the basal cells (± SE) in the epidermis of Large White pigs after irradiation with single doses of 15Gy (▲) and 20Gy (●) of X-rays. The open symbols represent the values for the regenerating cell colonies and the hatched area the density of cells (±SE) in unirradiated skin.

This finding has important implications for non-uniform exposure, since irradiation with intermediate energy β-emitters will leave a high proportion of reproductively viable epithelial cells within the canal of hair follicles, even after relatively high skin surface doses.

The main erythematous reaction and the associated epithelial response in areas exposed to β-rays for strontium-90 (E_{max} 2.2MeV) and thulium-170 (E_{max} 0.97MeV) are compared in Fig. 3. The responses of two representative areas are compared, irradiation was with sources of comparable size and with skin surface doses at the ~ED_{100} for moist desquamation, i.e. 40Gy and 130Gy for strontium-90 and thulium-170, respectively. In each instance the maximum level of erythema was seen at 3-6 weeks after irradiation and was classified as moderate to bright red (score B-C). Moist desquamation (score S or T) developed after 4-6 weeks, this was transient and a dry, scaly, desquamation was reported after 5-6 weeks (score R). Moist desquamation was more prolonged after strontium-90 irradiation despite the fact that these sites received a lower skin surface dose. The more rapid resolution of moist desquamation after thulium-170 irradiation, and the higher skin surface doses required to produce the same effect as for strontium-90, can be explained by the presence of a large number of surviving epithelial cells within the canal of hair follicles.

At doses above the ED_{100} for moist desquamation the basal layer of the epidermis was frequently found to be completely denuded of clonogenic cells; secondary ulceration, involving the loss of dermal tissue, tended to

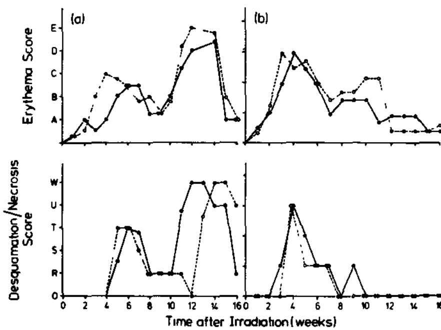

Figure 3: Time-course of changes in the severity of erythema and an indication of the presence or absence of desquamation and dermal necrosis after the irradiation of pig skin with surface doses representing the ED_{90} for moist desquamation from strontium-90 (a) or thulium-170 (b) i.e. 40Gy and 130Gy, respectively. Responses for individual fields from two pigs are illustrated which were assessed at weekly intervals (Erythema: A - minimal; B - moderate; C - bright red; D - dusky; E - mauve. Desquamation: R - dry; S - <½ and T - >½ the irradiated area showing moist desquamation. Necrosis: U - <½ and W - >½ the irradiated field).

develop in such situations as a consequence of both infection and trauma. Healing was by invasive fibrosis leading to scar tissue formation.

c) Late erythema

The late phase of erythema varies in its severity but at its maximum is characterised by skin with a dusky or mauve appearance. The incidence of these changes increases with dose after a threshold dose of ~15.4Gy of X-rays. The reaction is associated with the presence of oedema and studies of lymphatic clearance, after a single X-ray dose of 18Gy, have provided evidence for impaired lymph flow at 9 and 12 weeks after irradiation. Necrosis was found to develop in ~50% of sites after a dose of 20.70Gy, a dose at which all fields showed the distinct dusky/mauve erythema which is taken to be representative of vascular insufficiency. A comparison of the severity of this late phase of erythema (Fig. 3) and the associated depth dose curves for the two β-emitters strontium-90 and thulium-170 have identified the vessels of the deep dermal plexus as an important target cell population. This suggestion is supported by the histological appearance of the vessels in this region; occlused or partly occluded arterioles were a frequent finding.

d) Late skin atrophy

After irradiation with single doses from a 22.5mm diameter strontium-90 plaque dermal atrophy in pig skin has been shown to develop in two distinct phases (Fig. 4). The first phase developed between 14-20 weeks and the later phase >52 weeks after irradiation.

The mechanisms leading to these two phases of injury are still uncertain. It is probable that the pathogenesis of the first phase is similar to that proposed for the development of dermal necrosis over a similar time scale after higher doses. An alternative suggestion, that it might represent the loss of parenchymal fibroblasts, would not appear to be the case since any loss of fibroblasts follows the vascular changes. This suggests that fibroblasts are not a primary target cell population. The pathogenesis of the second phase of dermal thinning is even more obscure. However, degeneration of the smooth muscle cells of the tunica media of small arterioles and their replacement by hyaline material is seen, along with the presence of dilated telangiectatic capillaries, petechial haemorrhages and foci of necrosis.

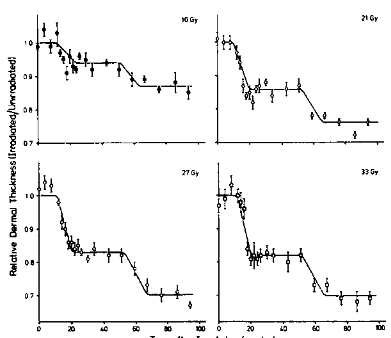

Figure 4: Time-related changes in the relative thickness (±SE) of the dermis of pig skin after irradiation with single doses of 10, 21, 27 and 33Gy from a 22.5mm diameter strontium-90 plaque.

EFFECTS OF HIGHLY NON-UNIFORM LOCALISED EXPOSURE

Localised non-uniform exposure of the skin may result from contact with highly radioactive particles, 'hot' particles, with a diameter of ≤2mm or from irradiation with very low energy β-emitters.

a) 'Hot' particle irradiation

The primary lesion resulting from irradiation with 'hot particles' of strontium-90 or thulium-170 is acute ulceration. The depth and size of the

ulcer will depend on the dose and the energy of the emissions from the particle. The full lesion develops within two weeks, well in advance of the time scale associated with the development of moist desquamation. This lesion was not found to be associated with 'hot' particle irradiation because the cell depletion caused by a loss of the reproductive capacity of basal cells was replaced rapidly by the migration of viable cells from outside the high-dose area. Prior to the development of ulcers, after doses of >100Gy (the dose averaged over $1.1mm^2$), a small pale circular area with a slight bluish tinge can be detected; this is frequently surrounded by a halo of erythema. Within a few days of irradiation histological studies have shown pyknosis of the nuclei of endothelial cells and fibroblasts in the papillary dermis and by 5-7 days after irradiation the papillary dermis is largely devoid of cell nuclei beneath the source although a paley staining epidermis remains intact. Ulceration is evident when the overlying epidermis separates from the necrotic dermis and is lost. Doses that are usually associated with producing the death of cells in interphase will produce acute ulceration. Shallow ulcers may be covered by a dry serum exudate, but this should not be confused with dry desquamation which, anyway, occurs over a longer time scale. Healed lesions leave a scar with the appearance of a small dimple.

b) Low energy β-irradiation

These investigations have been carried out using promethium-147 (E_{max} 0.22MeV) the depth dose profile of which is such that there is an ~80% reduction in dose across the layers of the epidermis. Histological studies carried out after promethium-147 irradiated have shown that for very high skin surface doses (>200Gy) the earliest detectable lesion is pyknosis of the cells of the upper viable layers of the epidermis after 3 days. Cell death within these layers initiates an inflammatory reaction with the subsequent total disruption of the epidermis after 7-10 days. This has the gross appearance of moist or dry desquamation (i.e. a dried serum exudate) which fully heals within 3-7 days, i.e. within ~20 days of irradiation. This is well before the normal time of appearance of classical moist desquamation which has a totally different underlying pathogenesis.

These investigations provide, for the first time, a detailed understanding of the types of reactions that may be encountered in the skin after exposures that are of relevance to radiation protection. This is of considerable benefit to this area of the European Communities Radiation Protection Programme, providing a better scientific basis for future investigations related to radiological protection and skin exposure.

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Title of the project no.: 2

The biological effects of non-uniform irradiation of pig skin

Head(s) of project:

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Dr. V. Sieber

I. Objectives of the project:

Considerable concern has been expressed as to the adequacy of the existing radiological protection guidelines as they apply to the exposure of the skin. Occupational exposures involving the skin are frequently non-uniform and can involve the irradiation of either a significant area of skin or a very small region, as in the case of contact with a highly radioactive particle ('hot particle'). To improve the existing radiological protection guidelines information is needed on the dose-related changes in both the early and late effects of radiation from sources of differing size, energy of emission, and dose-rate.

II. Objectives for the reporting period:

Studies have been carried out on the skin of pigs to determine the dose-related changes in the acute response from three β-emitters, strontium-90 (high energy), thulium-170 (intermediate energy) and promethium-147 (low energy). Irradiation has been with sources which varied in size from 0.1-40mm in diameter. Exposures from these sources were considered to be acute exposures, in that they had a maximum duration of ≤2 hours. For a 22.5mm diameter source of strontium-90 the effects of radiation dose-rate have been examined.

The effects of the area of tissue irradiated on the severity of late dermal atrophy has also studied at 2 years after irradiation from strontium-90 and thulium-170 sources.

III. Progress achieved:

In an extensive series of studies, dose-effect relationships have been established for the endpoints of early moist desquamation and late dermal atrophy after the irradiation of areas of skin of ≧19.5mm^2 and for acute ulceration and late dermal atrophy after 'hot particle' exposure. A 'hot particle' refers to a source of ≦2mm diameter. For irradiations involving the very low energy β-emitter, promethium-147, the dose-related incidence of acute epidermal necrosis was investigated for sources of differing size.

a) Moist desquamation

The dose-related changes in the incidence of moist desquamation, which develops after 4-6 weeks, were determined after the irradiation of pig skin with strontium-90 sources of 5-40mm in diameter. After an initial threshold dose the proportion of fields irradiated that developed moist desquamation increased with dose (Fig. 1). The ED_{50} values (±SE) for moist desquamation were derived from these curves and are listed in Table 1. In this table are also listed the associated ED_{10} (±SE) and estimated threshold doses obtained following irradiation with this range of source size. The ED_{50} declined markedly from ~70Gy from a 5mm diameter strontium-90 source to ~27Gy for a ≧22.5mm diameter source.

Perhaps of significance, from the view point of mechanisms, is the observation that the dose-effect curves for the 5, 11 and 15mm diameter sources were significantly shallower than those for the two larger sources. This implies a greater inhomogenicity in the cell populations irradiated with the smaller sources. This may reflect an increase in the stimulus for cell migration after higher doses from these sources. This would also help to explain the reduction in the source-size effect at the ED_{10} and at the estimated threshold doses for moist desquamation.

The irradiation of skin with a β-emitter of a significantly lower energy than strontium-90, thulium-170 (E_{max} 0.97MeV), would leave many reproductively viable basal cells within the irradiated area i.e. those basal cells situated in the canal of the hair follicle. In such a situation the migration of cells from the edges of an irradiated area would be expected to be of less importance in determining the response of skin to increasing areas to irradiation. The finding of a significantly reduced source-size effect and higher skin-surface-doses for both the ED_{50}, ED_{10}, and estimated threshold doses in pig skin after irradiation with thulium-170 sources of 5-19mm diameter (Fig. 2) provides major evidence

for the presence and importance of these viable clonogenic cells from within the canal of the hair follicles.

For strontium-90 sources of 22.5mm diameter the ED_{50} for moist desquamation was also found to vary markedly with dose-rate. This ranged from 27.3 ± 0.7Gy for doses given from the high dose-rate source to >100Gy for doses given at 0.01Gy/min (Table 2).

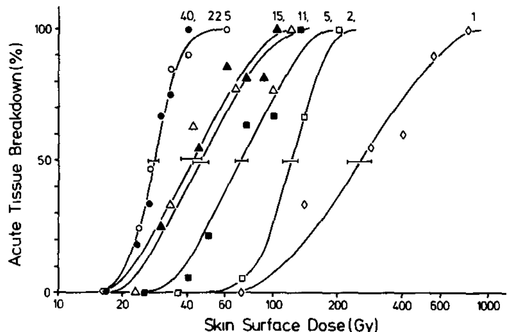

Figure 1: Dose-related changes in the percentage of skin sites developing moist desquamation after irradiation from 5-40mm diameter strontium-90 plaques and acute ulceration after irradiation from 1 or 2mm diameter 'hot particles'. The data are those of Hopewell et al. (1986) replotted by probit analysis. Error bars indicate ±SE on the ED_{50} values (● - 40mm; ○ - 22.5mm; ▲ - 15mm; △ - 11mm; ■ - 5mm; □ - 2mm; ◇ - 1mm).

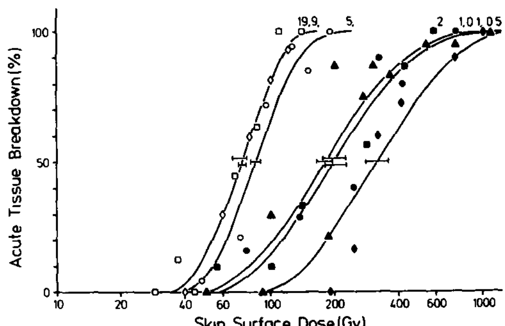

Figure 2: Dose-related changes in the percentage of skin sites developing moist desquamation after irradiation from 5-19mm diameter thulium-170 plaques and acute ulceration after irradiation from 0.1-2.0mm diameter 'hot particles'. The data are those of Hopewell et al. (1986) replotted by probit analysis and including a dosimetry correction for the 0.1-1.0mm diameter sources. Error bars indicate ±SE on the ED_{50} values. (□ - 19mm; ◇ - 9mm; ○ - 5mm; ▲ - 2mm; ● - 1mm; ◆ - 0.5mm; ● - 0.1mm).

Table 1 Variation in ED_{50} values (±SE), ED_{10} and estimated threshold doses for acute breakdown of the skin of pigs after β-irradiation

Isotope	Sources diameter (mm)	ED_{50} (Gy)	ED_{10} (Gy)	Estimated threshold dose (Gy)	Type of lesion
$^{90}Sr/^{60}Y$	40	28.6 ± 1.4[a]	20.1 ± 1.4	~17.5	
	22.5	27.3 ± 0.7	21.1 ± 1.2	~17.5	
	15	42.3 ± 4.8	21.0 ± 3.4	~17.5	MD
	11	44.8 ± 4.5	22.0 ± 3.2	~19.0	
	5	70.3 ± 5.0	34.4 ± 4.0	~25.0	
	2	119.3 ± 9.0	82.0 ± 10.1	~50.0	AU
	1	253.7 ± 31.0	104.2 ± 24.6	~70.0	
^{170}Tm	19	71.9 ± 5.7[a]	47.5 ± 4.2	~35.0	
	9	73.9 ± 2.6	48.8 ± 2.8	~35.0	MD
	5	85.8 ± 4.6	56.7 ± 3.9	~39.0	
	2	178.7 ± 14.9	77.5 ± 9.4	~50.0	
	1	201.9 ± 23.7	87.7 ± 12.6	~65.0	
	0.5	338.6 ± 35.6	147.1 ± 19.5	~85.0	AU
	0.1	197.4 ± 24.9	85.7 ± 13.1	~60.0	
		246.0 ± 37.8	106.8 ± 16.4	~70.0	
^{147}Pm	15	512 ± 33[b]	305 ± 41	~200	
	9	572 ± 35	366 ± 45	~200	
	5	615 ± 33	408 ± 40	~250	AEN
	2	717 ± 39	510 ± 40	~350	

[a]Dose at a 16μm depth, averaged over $1.1mm^2$
[b]Dose at the skin surface, averaged over $1.1mm^2$
MD - moist desquamation; AU - acute ulceration;
AEN - acute epidermal necrosis

Table 2. Variation in the ED_{50} (±SE) for moist desquamation in pig skin after irradiation with β-rays from 22.5mm diameter strontium-90 plaques of differing dose-rate

Dose Rate (Gy/min)	ED_{50} ±SE (Gy)
3.0	27.3 ± 0.7
0.102	36.8 ± 3.2
0.048	42.2 ± 2.0
0.023	66.6 ± 3.7
0.01	>100

b) Highly non-uniform irradiation

- Irradiation with poorly penetrating β-emitters

A comparison of the acute radiation responses of the skin to a low energy emitter source, such as promethium-147, with that of strontium-90 and thulium-170 is not meaningful because of the different biological responses they produce. The dose-effect curves for acute epithelial necrosis after promethium-147 irradiation are shown in Fig. 3. A small source-size effect was seen. However, this is of doubtful significance given the difficulties associated with the recognition of minor skin changes in very small areas after irradiation with the smaller sources.

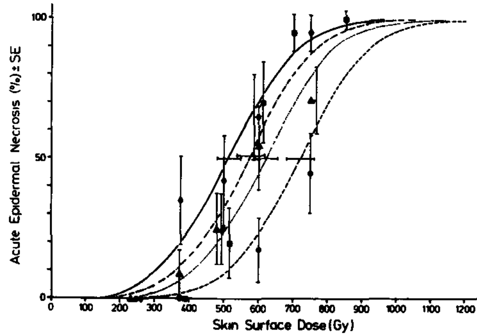

Figure 3: Dose-related changes in the percentage of skin sites developing acute epithelial necrosis (±SE) after irradiation from 2-15mm diameter promethium-147 plaques. (▲ - 15mm; ■ - 9mm; ♦ - 5mm; ● - 2mm).

The skin surface doses required to produce any effect after promethium-147 irradiation i.e. >200Gy would suggest that persons likely to be exposed to low energy β-rays of ~0.225MeV may warrant special consideration when setting dose limits.

- Irradiation with 'hot' particles

The dose-response curves showing the proportion of areas of skin that were exposed to β-irradiation from 'hot' particles of either strontium-90 or thulium-170 that developed acute ulceration are also illustrated in Figs. 1 and 2. The majority of these dose-effects were much shallower than those associated with the development of moist desquamation. This indicated that a six-fold increase in dose was associated with the change from a 10% incidence of acute ulceration (ED_{10}) to a 90% incidence. The doses quoted for these studies were measured with an extrapolation chamber with an equivalent window thickness of 16 μm with a collecting electrode with an area of $1.1mm^2$. The ED_{50} for acute ulceration from 'hot' particles of ≤1mm diameter was ~250Gy. Slightly lower ED_{50} values of ~120Gy and ~180Gy were obtained from 2mm diameter particles of strontium-90 and thulium-170, respectively (Table 1).

c) Late skin damage

For irradiations with intermediate and higher energy β-emitters late skin changes, which include dermal atrophy, may prove to be cosmetically the most unacceptable late normal tissue changes that could determine the dose-limit for radiological protection. Measurements of dermal thickness at 2 years after the irradiation of pig skin showed that significant dermal thinning was observed at doses that did not produce an early epithelial desquamation or acute ulceration (Figs. 4 and 5). However, threshold doses for atrophy of skin have still to be established for a severity of dermal thinning that might be considered to be cosmetically unacceptable. Extrapolation from data showing the severity of dermal thinning after irradiation from strontium-90 and thulium-170 would suggest a threshold-dose of ~10 and ~15Gy for the prevention of dermal thinning for this energy of β-emitters for sources of 5-22.5mm diameter (Figs. 4 and 5). These exposures were given at a high dose-rate.

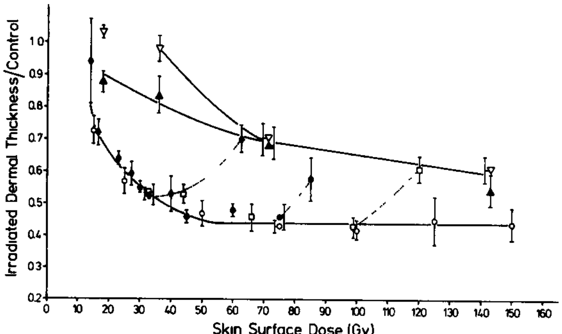

Figure 4: Relative reduction in dermal thickness (±SE) in pig skin 104 weeks after irradiation with strontium-90 β-rays from a 22.5mm (♦), 15 (●), 11 (□), 5 (○), 2 (▲) or 1 (▽) mm diameter source.

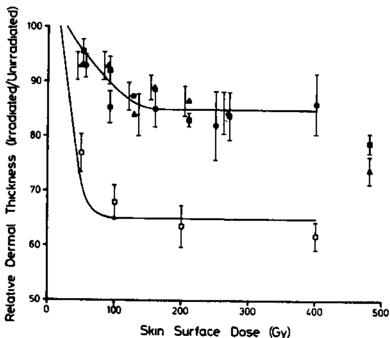

Figure 5: Relative reduction in dermal thickness (±SE) in pig skin 104 weeks after irradiation with thulium-170 β-rays from a 0.1 (■); 0.5 (▲); 1.0 (●) and 2.0mm (□) diameter source. The data for 5-19mm diameter source were equally well fitted by the line fitted through the 2.0mm diameter data points.

These data provide, for the first time, a rational guide for the development of improved radiological protection criteria for the skin. The information has provided a major contribution to both ICRP and NRCP task group reports relating to the problems of skin exposure. This provides perhaps the best evidence and of the relevance of the studies to the European Communities Radiological Protection Programme.

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Hopewell, J.W., Hamlet, R. and Peel, D.M. (1985). The response of pig skin to single doses of irradiation from strontium-90 sources of differing surface area. Brit. J. Radiol. 58, 778-780.

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-C-309-IRL

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Department of Genetics
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Head(s) of research team(s) [name(s) and address(es)]:

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Title of the research contract:

Rapid techniques for graft assessment following bone marrow transplantation designed for large scale use following accidental lethal exposure to radiation

List of projects:

1. Rapid techniques for graft assessment following bone marrow transplantation designed for large scale use following accidental lethal exposure to radiation

Title of the project no:

Rapid techniques for graft assessment following bone marrow transplanation designed for large scale use following accidental lethal exposure to radiation.

Head(s) of project:

Dr. Peter Humphries

Scientific staff:

Dr. Mark Lawler

I. **Objectives of the project:**

Our objectives have been to develop a most highly sensitive and rapid process for analysis of donor and recipient cells for use in the monitoring of bone marrow transplantations. Our process is based upon a series of high information-content DNA markers and the polymerase chain reaction. The process has been developed with the objective of assessing conditioning therapies (* total body irradiation) and the role of mixed chimerism in long-term survival, and as a means of rapid engraftment monitoring in an environmental crisis situation.

II. **Objectives for the reporting period:**

As I. above.

III. Progress achieved:

1. Introduction:

The occurrence of a major nuclear accident at the nuclear power station in Chernobyl on 26th April 1986 attracted major media coverage, particularly concerning the effect of such an accident on the health of the human population. Discussion of the incident also focussed on the types of therapy available to victims of such an accident. Several articles have been written on the pros and cons of using bone marrow transplantation (BMT) as a modality of therapy for victims exposed to radiation doses such as those experienced at Chernobyl (1,2). Hemopoietic stem cells are highly sensitive to the cytotoxic effect of ionizing radiation and at levels of >8Gy, toxicity to these marrow cells may be lethal. At present BMT is a successful treatment for a wide range of haematological malignancies, including leukaemia and aplastic anaemia (3), and is a logical treatment for victims of accidental total body irradiation who receive a sufficiently high dose that they are likely to die from marrow failure. While there would be problems associated with BMT in the Chernobyl situation (other radiation injuries, lack of donors, lack of previous experience) it may still benefit certain subsets of accident survivors. However, as in all BMT situations, it is important to be able to monitor the outcome of a transplant. This could be of particular importance in a crisis situation where decisions concerning therapy or a second transplant operation may have to be taken quickly to ensure survival of the patient.

In order to monitor a transplant successfully, it is necessary to have a marker which allows discrimination between donor and host cells. (1) To assess engraftment or rejection and (2) as an index of mixed chimerism (the coexistence of donor and host cells in a stable chimera following BMT). Classically, this has been achieved by the use of protein polymorphisms (red cell antigens, isoenzymes, immunoallotypes) or by karyotypic analysis (4-6). Recently the increasing use of DNA technology has allowed the sensitive monitoring of engraftment post BMT (7,8). This has been achieved by using DNA probes which detect neutral variation in the DNA of the donor and the host. This variation can be of two main types:

(a) Restriction fragment length polymorphisms (RFLPs). Here mutations in DNA create or destroy restriction enzyme sites.

(b) Minisatellites and variable number tandem repeats (VNTRs). Here the variation is in the length of tandemly repeated blocks of DNA.

While judicious use of such probes allows virtually all transplants to be monitored, the time for analysis, lack of sensitivity at very low levels of mixed chimerism and possible problems in the routine analysis in time limiting situations has prompted us to investigate new, more rapid and sensitive assays for assessment of chimerism post BMT.

Initially we developed a dot blot assay for assessment of sex mismatched transplants (9). This assay was based on the use of a DNA sequence that was present on the Y chromosome at a copy number approaching 2000. An assay was devised which permitted Y specificity to be achieved in a dot blot format. The highly repetitive nature of the probe allowed a high level of

sensitivity to be achieved. In the period of the present project we have been engaged in the development of rapid engraftment assays based on a recently developed technique, the polymerase chain reaction (PCR). This technique permits the rapid exponential accumulation of specific target DNA from a defined DNA template by an in vitro amplification reaction (10). We have adapated this technique initially to the analysis of sex mismatched transplants (11,12) but more recently the technique has been extended to the analysis of all transplant situations (13, Lawler et al., manuscript in preparation).

2. **Methodology**:

PCR is achieved by the use of synthetic oligonucleotides to prime a region of DNA for subsequent copying by a DNA synthesising enzyme called DNA polymerase. These synthetic sequences (called primers or amplimers) are chosen to compliment flanking regions of the target DNA. Under appropriate annealing conditions these primers bind to denatured DNA and act as a starting site for DNA polymerase, provided the necessary building blocks (nucleoside triphosphates) are present. Repeated cycles of DNA denaturation, primer annealing and DNA synthesis (extension) allows for exponential increase of the target DNA, as DNA synthesised in one cycle of amplification acts as a template for the next cycle.

(a) **Sex mismatched analysis**:

We have adapted this technique to the amplification of Y specific sequences. Primers which specify Y specific fragments have been synthesised on an Applied Biosystems DNA synthesiser (Model 391A "PCR MATE") (See Table 1). Bone marrow aspirates, peripheral blood samples or stored slide material is then prepared for analysis as follows. White cells are pelleted, resuspended in dH_2O and boiled for five minutes to lyse cells and release the DNA. (For stored slides, material is scraped off directly into dH_2O for subsequent lysis using a sterile scalpel blade). Cell debris is pelleted and an aliquot of the supernatant (containing the crude extract of DNA) is taken for PCR analysis.

Following PCR (detailed conditions are outlined in 11) the amplified material is assayed by electrophoresis through a 2% agarose gel. Fidelity of amplification is confirmed by digestion of the amplified fragment with a restriction enzyme and subsequent analysis on an agarose gel with known DNA molecular weight markers.

(b) **Sex matched transplants**:

Recently it has been discovered that the tandem repetitive block $(dC\text{-}dA)_n : (dG\text{-}dT)_n$ is a source of natural variation in human DNA which can be accessed using PCR (14,15). These blocks are called microsatellites and they show variation in the number of repeat blocks between individuals. Since there are 50,000 of these so-called "CA repeats" scattered throughout the genome, they represent a new source of natural polymorphism in human populations. PCR can be used to amplify and type the different variants (alleles) at various microsatellite loci throughout the genome, allowing markers to be generated which can be used to assess all transplant situations, regardless of sex. Primers are synthesised to bind to single copy sequences flanking these mirosatellites. PCR is performed as before, except that ^{32}P labelled dCTP is encorporated into the PCR product. The product is then electrophoresed on an 8% polyacrylamide denaturing gel and differences between individuals at these polymorphic loci are reflected in a different mobility after electrophoresis. This can by visualised by drying down the gel and exposing it to autoradiography for a short period of time. Several microsatellites may be typed simultaneously allowing many transplant situations to be assessed rapidly.

3. **Results and discussion**:

(a) **Sex mismatched analysis**:

Using the techniques as outlined above, chimerism was assessed in five patients following sex matched allogeneic BMT to assess the validity of the assay. As well as using PCR, all samples were also tested by karyotyping of bone marrow cells and dot blot analysis of blood and bone marrow. While the results of Karyotypic analysis suggested a complete chimerism in four patients and the presence of a mixed chimera in one patient, PCR also detected a mixed chimeric state in a second patient which has now persisted to Day 925 post transplant and which is still undetected by karyotypic analysis. The patient in which mixed chimerism was detected by PCR and karyotyping subsequently relapsed and the incidence of mixed chimerism and its subsequent effect (if any) on relapse or survival is currently being addressed in a long term study on 33 patients, sex mismatched and sex matched (Lawler et al, manuscript in preparation). The sensitivity of the assay of sex mismatched situations was such that a positive male signal could be obtained from 500-5000 male cells by direct gel analysis and this could be reduced to 5-50 cells by the use of autoradiography following primer labelling or encorporation of label into the PCR product. While DNA extraction for Southern analysis can be time consuming and laborious we have reported here a rapid lysis method and direct PCR which allows samples to be analysed in < 24 hours. PCR allows rapid enrichment of source material providing adequate amounts of high quality material for analysis.

(b) **Sex matched analysis**:

Recently we have extended this technique to the analysis of sex matched material by amplifying microsatellites as described in the section on Methodology. We have demonstrated the usefullness of this technique by showing that all of 33 donor recipient pairs may be monitored successfully using a panel of

oligonucleotides as outlined in Table 1 (13). This technique is quite easy to perform and does not require purification of high quality DNA. This combined with the lack of need for time consuming Southern analysis and ease of reading autoradiographs (which could be automated to computer gel reading) makes us feel that this method is even superior to minisatellite analysis in the assessment of chimerism post transplant.

The application of the techniques outlined in this report to stored slide material means that retrospective as well as prospective studies can be performed. Recently we have established a collaboration with the University of Minnesota to look at chimerism in a large number of patients at that transplant centre solely by the analysis of stored slide material. To date 20 patients have been assessed and we are submitting the data accrued in this study to the American Society of Haematology Meeting in Boston in December 1990. Also a collaboration will begin in Spring 1990 in a prospective study on the assessment of chimerism in severe aplastic anaemia. This will be done in collaboration with the EBMT Working Party on Aplastic Anaemia.

The sensitivity and rapidity of the techniques described above will allow many samples to be analysed simultaneously and with great accuracy, parameters which could be of major significance in the management of transplanted victims in a crisis situation following an accidental release of ionizing radiation into the environment. The techniques also allow a thorough investigation of the natural progression of a bone marrow transplant and allow us to answer questions concerning the incidence of mixed chimerism and its subsequent effect on graft relapse rejection or overall engraftment and survival.

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-C-069-B

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Title of the research contract:

Morphological and cytogenetical studies on the sensitivity of the mammalian embryo to low doses of radiation.

List of projects:

1. Morphological and cytogenetical studies on the sensitivity of the mammalian embryo to low doses of radiation.

Title of the project no.: 1

Morphological and cytogenetic studies on the sensitivity of the mammalian embryo to low doses of radiation

Head(s) of project:

P. Jacquet

Scientific staff:

P. Jacquet, S. Grinfeld, L. Baugnet-Mahieu, A. Léonard

I. Objectives of the project:

In vitro culture of embryos represents a simple model system to analyse very precisely the modifications of radiosensitivity occurring during the preimplantation period. The one-cell embryo has been little studied and results obtained so far showed that this stage is particularly radiosensitive. However, the techniques utilized for these investigations were very different and implied, for example, in vitro or in vivo fertilization and irradiation, use of natural or hormone-stimulated ovulation. In addition, hybrid or inbred strains of varying radiosensitivities were used. Such variations in the experimental conditions could explain, at least partially, important discrepancies reported between laboratories. In this project, we intended to study the influence of these factors, and the mechanisms by which they can modify the response of the embryo to X-irradiation.

II. Objectives for the reporting period:

This report is a summary of the main results obtained during the period 1985-1989. As underlined in the discussion, some studies are still in progress. In order to spare space, the methodology has been restricted to the strict minimum necessary to the comprehension of the experiments. Detailed data on the techniques used and on the results will be found in the publications of which the references are mentioned at due places.

III. Progress achieved:

Methodology

1. Influence of the strain on the radiosensitivity of the mouse zygote : studies on the "one-cell block"

1.1. The "one-cell block", as an unusual radiation-induced G2-arrest. Time-dependence, dose-dependence, and its specificity (refs. 1-2).

It is generally believed that embryonic mortality induced by irradiation of the preimplantation stages occurs mainly near the time of implantation. However, results obtained in our laboratory suggested a high loss before the first cleavage, when BALB/c zygotes were irradiated at the pronuclear stage. To clarify these observations, BALB/c females were induced to superovulate by i.p. of 5 i.u. PMS and 5 i.u. hCG 48 hours apart. Such hormonal treatment induces superovulation about 12 hours after hCG injection. Females were caged overnight with males of the same strain and irradiations with 1 Gy of X-rays were performed each hour during the 12 hours following fertilization. Females were killed on the following morning and the proportion of eggs which had cleaved were noted. 2-cell embryos were cultured for 4 days, at the end of which the proportion of those which had reached the blastocyst stage were also noted. Development to the 2-cell stage and to the blastocyst stage were combined to calculate the LD50 value for the time of maximal radiosensitivity, after application of different doses of X-rays.
Our studies were devoted to the characterization of the block effect found in a number of eggs irradiated at the pronuclear stage. This effect will be referred to as the "one-cell block". The eventual existence of the "one-cell block" was investigated in four other strains (T-stock, CF1, AKR and C57Bl). In addition, investigations on the importance of the maternal genotype for this effect were performed by irradiation of pregnant females from different crosses involving the BALB/c strain (♀ BALB/c x ♂ F1 (♀ BALB/c x ♂ C57Bl) ; ♀ F1 x ♂ BALB/c ; ♀ F1 x ♂ F1). The fact that the "one-cell block" is a particular form of G2-arrest was verified by determination of the DNA content in a number of pronuclei. For this purpose, irradiation was performed at 20 hours after hCG (time of maximal sensitivity for the "one-cell block"), and BALB/c eggs were collected and fixed between 30 hours (i.e. just before the presumed first cleavage) and 50 hours after hCG, before being analyzed by cytofluorometry.

1.2. Analysis of polypeptide synthesis and phosphorylation in blocked eggs (refs. 3,4,6)

The "one-cell block" could possibly result from an action of radiation on some critical protein(s) needed for division. A study of polypeptide synthesis and phosphorylation during the first 40-45 hours of development was therefore undertaken in control and irradiated eggs. Details on the techniques used for these studies may be found in refs. 3, 4 and 6. For these studies, BALB/c eggs were either used as controls, or *in vivo* irradiated with 2.5 Gy at 20 hours after hCG (with that dose, all embryos are blocked). In addition, a complementary study was performed on F1 eggs, either untreated or irradiated *in vivo* with 2.5 Gy (no block effect in that strain) or 8 Gy (almost all blocked) at 20 hours after hCG.

1.3. Influence of caffeine on the "one-cell block" (ref. 5 + results to be published)

Since caffeine is known to reverse G2-arrest in many cell types, we also tested its action on irradiated, blocked eggs. Those were incubated at different times after irradiation of the mothers, in a medium containing various concentrations of caffeine. The influence of caffeine on G2-arrest and embryonic development was determined.
The question of the necessity of protein synthesis for the action of caffeine was tested by incubating eggs with caffeine and cycloheximide according to various schemes. For this purpose, synchronous populations of eggs were used (mating possibility reduced to 2 hours) since, even in control eggs, protein synthesis is necessary for the progression to mitosis until one or two hours before it.

1.4. Relationship between the "one-cell block" and chromosome damage (ref. 5)

According to some authors, chromosome damage is, directly or indirectly, the cause of G2-arrest. Thus, we studied chromosome damage in zygotes able to escape G2-arrest after irradiation at different times of the cell cycle, and we compared it with the chromosome damage found in zygotes cleaving after G2-arrest or which would be blocked but were forced to cleave at the right time by incubation with caffeine.

1.5. Irradiation and adaptive response in the mouse zygote (to be published)

Human lymphocytes exposed to very low doses of radiation (1 cGy) in G1, S or G2, are less susceptible to the induction of cytogenetic damage by subsequent high doses of X-rays. This has been attributed to the induction of a repair mechanism that causes the restitution of X-ray induced chromosome breaks. The possibility of an adaptive response was also investigated in the BALB/c zygote for two important reasons :

1) if chromosome damage is partially implied in the radiation-induced G2-arrest, improvement of its repair could diminish the proportion of blocked zygotes, or the duration of the block ;

2) Embryos escaping the "one-cell block" undergo at least 4 divisions before dying from the morula stage. We and others clearly showed that this non-specific preimplantation mortality is the consequence of chromosomal injury, leading to an elimination of genetic material during the successive divisions.
If doses as low as 1 cGy or even less can protect the zygote from a second, normally lethal dose, and allow its survival although a number of radiation events have taken place in the genome of the cell which functions as stem cell, the long-term consequences of this may be very important and require extensive investigations.

For these experiments, very synchronous populations of embryos were used. Three groups of pregnant females were constituted : 1) controls ; 2) females receiving 1 cGy 0.5 hours after fertilization and 1.5 Gy 7.5 hours later ; 3) females receiving only 1.5 Gy 8 hours after fertilization.

Influence of the treatment on the "one-cell block" and on the development of the embryos escaping it were examined.

1.6. One-cell block and irradiation with low doses (to be published)

Embryos given low doses of X-rays were collected a few hours before the presumed division, and transferred in culture medium. At each control, cleaved embryos were separated from the other ones, and their development was followed to the blastocyst stage.

2. Influence of various methodological factors on the radiosensitivity of the mouse zygote (ref.8)

These experiments were undertaken to investigate the influence of some methodological factors on the radiosensitivity of the mouse zygote. The factors studied were 1) the use of natural or hormone-stimulated ovulation (5 i.u. or 1 i.u. gonadotropins), 2) the procedure followed for fertilization : mating overnight, or only during a short period in the morning after all oocytes have been ovulated, <u>in vitro</u> fertilization, 3) the type of irradiation, i.e. <u>in vivo</u> or <u>in vitro</u> irradiation. The radiosensitivity of the BALB/c zygotes was estimated under the different experimental conditions by measuring their ability to cleave and to further develop to the blastocyst stage, after irradiation with different doses of X-rays at the pronuclear stage. Development to the 2-cell stage and to the blastocyst stage were combined to obtain the surviving fractions and these values were used to calculate the LD50 values. These were graphically estimated from the best fitting dose-response curves.

<u>Results</u>

1. Influence of the strain on the radiosensitivity of the mouse zygote : studies on the "one-cell block"

1.1. The "one-cell block", as an unusual radiation-induced G2-arrest. Time-dependence, dose-dependence, and its specificity.

Two types of effect were found in BALB/c embryos, depending on the time of irradiation during the first cell cycle. When irradiation with 1 Gy was delivered between 14 and 21 hours after hCG injection, eggs which had cleaved at the right time developed normally in culture up to the morula stage, where a high mortality occurred. On the other hand, when irradiation was performed between 17 and 24 hours after hCG, a high proportion of the eggs did not cleave at the right time, remaining blocked at the one-cell stage ("one-cell block"). The time of maximal sensitivity for this effect was found to be 20 hours after hCG, i.e. 8 hours after superovulation and fertilization. After different doses of X-rays applied at that time, the proportion of eggs which remained blocked at the one-cell stage increased linearly with the dose administered, from 0.25 to 2 Gy, at which dose more than 90 percent of the eggs were blocked. The block effect was not irreversible : after 1 Gy, about 30-40% of the blocked eggs recovered partially, cleaved with a delay of about 20 hours, but died soon thereafter.
When both types of effect of radiation (block at the one-cell stage and mortality at later stages) were combined, the time of maximal

radiosensitivity during the first cell cycle was also found to be 20 hours after hCG, with an LD50 value of 95 cGy for development to the blastocyst stage.
Although BALB/c zygotes were particularly sensitive for the "one-cell block", it was also found in CF1 zygotes, but the doses of X-rays needed were very high. Other strains tested were not sensitive for the "one-cell block". Furthermore, different crosses between BALB/c and F1 (♀ BALB/c x ♂ C57Bl) mice showed that the "one-cell block" is determined by the maternal genotype, resulting most probably from a primary action on a radiosensitive factor appearing 5 hours after superovulation, and synthesized from components accumulated in the cytoplasm during oogenesis. Cytofluorometric analysis of the pronuclear DNA content of uncleaved eggs showed that DNA synthesis was unaffected by X-irradiation, and that they were blocked in G2 phase of the first cell cycle. Consequently, the "one-cell block" constitutes a particular form of the so-called "G2-arrest".

1.2. Analysis of polypeptide synthesis and phosphorylation in blocked eggs

Results of detailed studies on protein synthesis during the first and second embryonic cell cycles showed that blocked BALB/c eggs underwent the same modifications in protein synthesis as control eggs of the same age, except during first mitosis, for three polypeptide sets of 30, 35 and 45 kilodaltons molecular weight. The most remarkable difference between them was the appearance in cleaving controls of 3 spots at 35 kilodaltons that were absent in blocked irradiated eggs. The same difference was observed in F1 eggs which received 8 Gy of X-rays, a dose leading to a block similar to that observed in BALB/c eggs given 2.5 Gy, whereas in F1 eggs irradiated with 2.5 Gy, a dose insufficient to block them, these 35 kd polypeptides were indeed present. After presumed division, biochemical modifications typical of the 2-cell stage (activation of the embryonic genome, synthesis of new polypeptides, disappearance of maternal proteins) occurred in blocked eggs, as they did in control 2-cell embryos. In addition, results of incubation with (^{32}P) showed that the first mitosis specific polypeptides which were lacking in blocked eggs resulted from post-translational modifications, involving phosphorylations. This suggests a link between the inability to progress to mitosis and an absence of specific phosphorylation.

1.3. Influence of caffeine on the "one-cell block"

Eggs were incubated immediately after irradiation of the mothers, in medium containing various concentrations of caffeine. With 2 mM caffeine, the G2-arrest induced by X-rays was almost completely suppressed. However, most eggs which had been induced to cleave without delay died during the subsequent stages, only a small proportion of them being able to reach the blastocyst stage. Eggs were incubated in 2 mM caffeine during different periods following irradiation. Results showed that caffeine must be present during the period of normal first cleavage (i.e. after 30 hours after hCG) to suppress the G2-arrest. Thus, caffeine clearly restores a process which immediately precedes division, but does not occur in embryos irradiated 10 hours earlier, with sufficient doses of X-rays. Incubations of very synchronous populations of eggs (possibility of mating limited to 2 hours) with caffeine and cycloheximide showed that 1) synthesis of

proteins occurs after the CH-TP in irradiated eggs (CH-TP : cycloheximide transition point, i.e. the time at which all protein synthesis normally needed for mitosis has been completed in control embryos, incubation with cycloheximide being then without effect on division) ; 2) when sufficient time is allowed for such synthesis to occur, addition of caffeine can allow to some extent recovery from the "one-cell block", even in the presence of cycloheximide.
Since the action of caffeine is restricted to the period of normal first cleavage, we concluded that caffeine could act by restoring, directly or indirectly, the post-translational modifications of polypeptides which, apparently, play an important role in the processes leading to division, an hypothesis which was also supported by the results from two-dimensional electrophoresis.

1.4. Relationship between the one-cell block and chromosome damage

Results from our cytogenetic studies performed on eggs cleaving at the right time showed that in such eggs, the proportions of chromosome aberrations varied in function of the time of irradiation, showing clear relations with the varying rates of lethality occurring from the morula stage. On the other hand, eggs undergoing a G2-arrest and analyzed in late cleavage, showed more chromosome damage than those escaping it, and similar results were obtained in eggs forced to cleave at the right time by caffeine. However, different elements also suggest that chromosome aberrations are surely not the only cause of G2-arrest and this assumption was still indirectly reinforced by the studied reported hereunder.

1.5. Irradiation and adaptive response in the mouse zygote

Preliminary results indicated that 1) irradiation of the zygotes by 1 cGy followed by 1.5 Gy 7.5 hours later influences neither the proportion of blocked eggs nor the duration of the one-cell block ; 2) in contrast, such treatment could confer a relative protection to those embryos escaping the one-cell block which showed a development quite similar to that of control embryos up to the blastocyst stage. This observation, which could be very important, must however be taken with caution, due to the still insufficient number of embryos observed.

1.6. One-cell block and irradiation with low doses

Results of these experiments showed that 10 cGy constitutes apparently the limit for induction of the one-cell block. In contrast with the results obtained after doses of 1 Gy or more, where the delay was always equivalent to one entire cell cycle, large individual differences were found after application of lower doses of radiation. Interestingly, eggs suffering even a slight delay were unable to survive up to the blastocyst stage, whatever the dose administered.

2. Influence of various methodological factors on the radiosensitivity of the mouse zygote

The results of our studies suggested that the protocols used for mating and fertilization probably have a greater influence on embryonic survival following irradiation than the use of gonadotropins to stimulate

ovulation. Highest degree of synchronism in the development of the embryos is achieved by restricting mating possibility to a short period or by using *in vitro* fertilization. Very low values of LD50 were obtained under such synchronous conditions (50-80 cGy), confirming the high radiosensitivity of the mouse zygote at the early pronuclear stage. Comparison between the effects of *in vivo* and *in vitro* irradiation did not permit the unequivocal conclusion of a greater radiosensitivity of the embryo *in vitro* than *in vivo*.

Discussion

The aim of the present project was to investigate the influence of various factors (strain, methodological factors) on the response of the preimplantation embryos to X-irradiation.

The BALB/c zygote has been found to be a very useful model to study the mechanisms of the radiation-induced "one-cell block". This constitutes a particular and very pronounced form of the so-called "G2-arrest" which, although described and investigated in cultured cells since more than 30 years, is still not clearly understood. A lot of work remains to be done on the BALB/c zygote as well as on other cells, before the mechanisms of G2-arrest become completely clarified. However, some conclusions may already be drawn from the work performed to date.

The radiation-induced G2 arrest has been considered by some authors as a passive consequence of a damage to a proteinaceous target whose integrity would be a prerequisite for progression to mitosis. Recent results suggest, however, that the restitution of such a division protein, or more broadly, repair of the lesion(s) responsible for G2-arrest is not an absolute requirement for G2 cell progression. This conclusion is based on the fact that, in the presence of caffeine, the requirement of irradiated CHO cells for protein synthesis is alleviated, suggesting that the mechanisms responsible for G2 cell progression actually remain intact in these cells (Rowley, Int. J. Radiat. Biol. 48, 811-820, 1985). According to Rowley, G2-arrest could then be an active response to irradiation, triggered by defects not directly concerned with progression, but presumably highly advantageous for the cell to repair before mitosis.

Other elements support the hypothesis of an active response. Indeed, recent studies on CHO cells strongly suggest that the primary target for G2-arrest should be located in the nuclear membrane (Schneidermann and Hofer, Radiat. Res. 84, 462-476, 1980) and in our experiments, the "one-cell block" cannot be induced, before 17 hours after hCG, a time corresponding to the formation of the pronuclei in the zygotes. If the primary target is located in the nuclear membrane, G2-arrest or, at least, the "one-cell block", should constitute an active response to irradiation. Indeed, it has been clearly shown that early development of the mouse egg occurs independently of the embryonic genome until the 2-cell stage, and neither physical nor chemical enucleation affects any of the molecular changes that have been detected before the early 2-cell stage. Experiments involving irradiation and replacement of the pronuclei by others from unirradiated zygotes will help to resolve conclusively this important point. If such zygotes suffer no "one-cell block", the target should be in the pronuclei and the one-cell block (and probably, more broadly, the G2-arrest) should be an active response to irradiation.

It must be noted that some results from our studies also support the hypothesis of a passive consequence. Indeed :

1) Synthesis of critical (?) proteins was found to occur after the CH-TP in irradiated embryos. Such protein synthesis is insufficient, per se, to induce the division of blocked embryos, but is necessary to allow the recovery from G2-arrest in the presence of caffeine.
2) Studies performed with doses of 1 or 2 Gy of X-rays showed that zygotes cleaving after G2-arrest carry multiple chromosome aberrations and die after a few more divisions.
3) Studies realized with much lower doses showed that zygotes undergoing even a slight division delay are also unable to reach the blastocyst stage.

Whatever the response, active or passive, we think that the absence of division, at least in the mouse zygote, could be indirectly linked to an absence of modifications of critical polypeptides, occurring at a post-translational level during the mitosis. This is supported by the absence of specific phosphorylations which was evidenced in the blocked zygotes at the normal time of mitosis and which, with the absence of the second round of replication, constituted the only visible qualitative difference with control embryos, up to the end of the second cell cycle.
One may imagine that the phosphorylation of specific polypeptides, occurring after a chain of reactions, should require a certain treshold of critical protein(s), a condition not satisfied in blocked zygotes despite the additional synthesis occurring after the CH-TP. Caffeine could act by forcing the process of phosphorylation of mitotic polypeptides, at the end of the chain of reactions.
The particular sensitivity of the BALB/c strain to the "one-cell block" could result form a deficiency or a lack of expression of components accumulated in the cytoplasm of the oocyte during oogenesis and which are required to repair radiation damage to the cell.
Taking into account all results obtained to date, we think that the "one-cell block" and more broadly, the radiation-induced G2-arrest, are certainly not a simple consequence of chromosome damage.

Among the methodological factors possibly affecting the response of the embryo to irradiation, the use of superovulating hormones has often been evoked. Our studies clearly showed that, in fact, these hormones do not increase the radiosensitivity of the mouse zygote. On the other hand, our results also suggest similar radiosensitivities of the zygote *in vitro* and *in vivo*.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

- Laboratoire de Biologie Générale, Université Paul Sabatier, Toulouse (France)
- Institut für medizinische Strahlenphysik und Strahlenbiologie, Universitätsklinikum Essen, Essen (F.R.G.)

V. Publications:

Publications in Scientific Journals

(1) S. Crinfeld and P. Jacquet, Effets des rayons X sur des oeufs de souris BALB/c irradiés durant le stade indivis.
- C.R. Soc. Biol., 180, 207-211 (1986).

(2) S. Grinfeld and P. Jacquet, An unusual radiation-induced G2 arrest in the zygote of the BALB/c mouse strain.
- Int. J. Radiat. Biolo., 51, 353-363 (1987).

(3) S. Grinfeld, J. Gilles, P. Jacquet and L. Baugnet-Mahieu, Late division kinetics in relation to modifications of protein synthesis in mouse eggs blocked in the G2 phase after X-irradiation.
- Int. J. Radiat. Biol., 52, 77-86 (1987).

(4) S. Grinfeld, J. Gilles, P. Jacquet and L. Baugnet-Mahieu, Modifications post-traductionnelles de certains polypeptides en relation avec la première mitose chez l'oeuf de souris BALB/c.
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- Roux's Arch. Dev. Biol., 197, 302-304 (1988).

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- Int. J. Radiat. Biol., 55, 719-720 (1989).

(8) P. Jacquet and S. Grinfeld, Influence of some methodological factors on the radiosensitivity of the mouse zygote.
- Teratology, submitted for publication.

Communications

P. Jacquet, Utilisation des techniques de culture *in vitro* pour l'étude des effets des mutagènes chimiques et physiques sur l'embryon préimplanté. CEN Mol, Dept. Radioprotection, 1985.

P. Jacquet and S. Grinfeld, Studies on the ability of different factors to modify the radiosensitivity of the early mammalian embryo. Europ. Soc. Radiat. Biol., 19th meeting, Prague, 1985.

S. Grinfeld and P. Jacquet, Existence of a particular radiation-induced G2 arrest in the mouse egg of the BALB/c strain. Europ. Environ. Mutagen Soc., 16th meeting, Brussels, 1986.

P. Jacquet and S. Grinfeld, Studies on the block effect induced in the BALB/c mouse zygote by X-rays. Joint meeting of Association for Radiation Research and Netherlands Radiobiological Society, Oxford, 1986.

P. Jacquet, Report on the research performed at Mol on the effects of radiation on preimplantation embryos. Meeting of the subgroup Embryology of EULEP (European Late Effects Project Group), Freiburg, 1987.

S. Grinfeld, P. Jacquet, J. Gilles and I. Baugnet-Mahieu, One-cell mouse embryo : a model for studies on G2 delay. Int. Cong. Radiat. Res., 8th Cong., Edinburgh, 1987.

P. Jacquet, Overview of the recent data on the radiosensitivity of the mammalian embryo during the preimplantation period (plenary session, invited lecture). Joint meeting of the Netherlands, Belgian and Swedish Societies for Radiobiology, the British Association for Radiation Research and the Radiobiology Committee of the British Institute of Radiology, Noordwijkerhout, 1988.

P. Jacquet and S. Grinfeld, Studies on the mechanisms of X-ray induced G2-arrest in the mouse zygote. European Teratology Society, 16th meeting, Baveno, 1988.

P. Jacquet, Report on the research performed at Mol on the effects of radiation on preimplantation embryos. Meeting of the subgroup "Embryology" of EULEP, Freiburg, 1989.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor:

Contract no.: BI6-C-065-F

Centre International
de Radiopathologie
B.P. n° 34
F-92260 Fontenay-aux-Roses

Head(s) of research team(s) [name(s) and address(es)]:

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Telephone number: 1-46.54.49.29

Title of the research contract:

Non-stochastic effects of irradiation in man: diagnosis, prognosis and treatment of acute radiation injury.

List of projects:

1. Biophysical, biochemical and cytological diagnosis of damage to skin and underlying tissues after accidental exposure. Improvement of therapeutic protocols.
2. Biological indicators of global irradiation.

Title of the project no.:

Projet 1

Head(s) of project:

Scientific staff:

Dr R.GONGORA
Dr V.MITZ

I. Objectives of the project:

Le contrat de recherche B16065-F portait sur les effets non stochastiques de l'irradiation de l'homme: diagnostic, pronostic et traitement des irradiations aigües accidentelles

Le projet n°1 concernait le diagnostic et le traitement des radiolésions cutanées: évaluation biophysique, biochimique et biologique des dommages à la peau et aux tissus sous-jacents après irradiation accidentelle; amélioration des protocoles thérapeutiques

II. Objectives for the reporting period:

III. Progress achieved:

Les irradiations partielles aigües ont en règle générale une origine accidentelle; aussi sont-elles relativement rares.

Chaque accident relève de circonstances exceptionnelles, qui lui sont propres. Aussi les paramètres d'exposition diffèrent d'un accident à l'autre.

Il s'ensuit que les manifestations cliniques sont multiples et variées.

Dans ces conditions, il s'avère difficile de recueillir les données suffisantes pour effectuer une étude systématique de la sémiologie clinique et des formes évolutives de la maladie et elles sont souvent mal connues. Cette étude toutefois a pu être entreprise à l'Institut Curie, où un grand nombre de sujets ont été examinés et traités.

Une des caractéristiques de l'évolution des irradiations partielles aigües est de comporter dans la plupart des cas une phase de latence clinique qui peut induire en erreur le médecin non averti.

D'autre part, l'évolution clinique est étroitement dépendante de la quantité d'énergie délivrée; aussi est-il indispensable de disposer d'examens paracliniques susceptibles de fournir des renseignements dosimétriques pendant la phase infraclinique.

Enfin, sur le plan thérapeutique, d'une part il y a carence de traitement étiologique, d'autre part le traitement symptomatique n'est pas parfaitement codifié; il souffre en particulier de la méconnaissance fréquente de la pathologie potentielle au moment où le médecin examine le sujet et prend les décisions thérapeutiques, chirurgicales notamment.

Systématisation des manifestations cliniques, mise au point d'investigations paracliniques, recherche de traitement étiologique et codification de la thérapeutique systématique constituent les trois principaux axes de recherche.

A. METHODOLOGIE

Les études ont porté, sur des patients ayant subi des irradiations partielles uniques accidentelles, sur certains malades traités localement par radiothérapie ainsi que pour certains investigations non dommageables sur des sujets normaux.

Les modalités d'irradiation partielle accidentelle offre un spectre particulièrement intéressant compte tenu de la diversité des sources et de la variété des paramètres spatiaux-temporels. On peut en particulier classer les rayonnements incidents en: photons x de faible énergie (2 à 30 Kev, 20 à 50 Kev); photons γ d'Ir 192 ($\approx$ 300 Kev), photons γ de Co 137 ($\approx$ 600 Kev) et de Co 60 ($\approx$ 1, 2 Mev).

Les investigations diagnostiques ont fait appel aux méthodes cliniques et paracliniques suivantes:

Sémiologie clinique
Thermographie à caméra infrarouge
à caméra microondes
Scintigraphie vasculaire
osseuse (traceur MDP-TC 99m)
Capillaroscopie: microscope + système vidéo

Les procédés thérapeutiques ont fait appel à des méthodologies médicales et chirurgicales concernant:

L'inflammation
Les infections
Les donneurs
Les nécroses

B. RESULTATS

Ils portent sur les diagnostic et le traitement des irradiations partielles accidentelles compte tenu des modalités de surexposition.

I. SYSTEMATISATION DE LA SEMIOLOGIE CLINIQUE

La sémiologie clinique des irradiations partielles aigües est extrêmement riche; de nombreux paramètres conditionnent sa diversité; ce sont essentiellement le niveau de dose , la

distribution chronologique et topographique de la dose, mais aussi les caractères physiques de la source (nature et énergie des rayonnements). L'évolution aussi est influencée par ces paramètres. On peut ainsi décrire un certain nombre de formes cliniques:

- des formes évolutives:
  - forme limitée, une épithéliite exsudative
  - forme avec endothéliite vasculaire
  - formes contractées
- formes liées aux caractères physiques de la source
- formes liées aux modalités chronologiques d'exposition
- formes liées au volume et à la topographie
- formes associées à une irradiation globale
- formes avec induction de cancer

II. THERMOGRAPHIE

2.1. Sujets normaux:

Les investigations ont porté essentiellement sur la main normale, vu la fréquence des irradiations palmaires. Elles ont fourni des informations sur:

- la température de base
- la carte thermique de la main
- l'influence du sexe
- les variations physiologiques
- le rôle du tabagisme
- les pathologies associées

2.2. Sujets irradiés accidentellement

Les résultats essentiels ont été obtenus concernant les niveaux thermiques, les caractères topographiques, la chronologie des réponses, les relations avec la dose, les conséquences cliniques et le bilan préchirurgical.

2.2.1 Niveaux thermiques

- seuil
- gradient minimal
- gradient maximal
- incidence du stress en fonction de la topographie
- effet écran des phlyctènes

2.2.2 Caractères topographiques
- corrélations avec la clinique
- évaluation de l'étendue potentielle des lésions
- gradient de dose étalé (source nue)
- gradient de dose étroit (collimation)
- effet d'écran des phlyctènes
- phénomènes d'accompagnement

2.2.3 Chronologie des réponses
- introduction
- précocité infraclinique
- fugaces
- tardives
- poussée
- long terme:
  - normalisation
  - hyperthermie
  - hypothermie
- physiopathologie

Relations avec la dose
- similitude de présentation
- apport à la dosimétrie physique relative
- gradient thermique - correspondance dosimétrique

Conséquences cliniques

Bilan préchirurgical

III. SCINTIGRAPHIE VASCULAIRE DES MAINS

Les paramètres étudiés sont:
- les rapports de concentration du traceur côté irradié/côté sain aux différents temps indiqués ci-dessus,
- les rapports de pentes des pics du premier passage vasculaire,
- les index de volume vasculaire, calculés pour chaque doigt,
- les profils longitudinaux montrant la répartition topographique du traceur au niveau des doigts

Un groupe témoin a été examiné, comprenant des patients pour lesquels, à l'occasion de scintigraphies osseuses demandées dans le cadre de leur bilan, le passage vasculaire du traceur au niveau des mains est enregistré.

Les résultats montrent des différences très significatives entre mains irradiées et mains non irradiées, ainsi qu'entre mains irradiées et les mains du groupe témoin. Ils mettent en évidence des augmentations importantes du débit vasculaire au niveau des territoires irradiés.

IV. CAPILLAROSCOPIE

Les sujets ont été examinés au niveau des doigts. On a obtenu, dans le cas de surexposition des doigts, une réponse directement liée à l'exposition; dans le cas d'exposition des mains, une réponse indirectement liée à l'irradiation mais cependant intéressante.

On a notamment observé:
- une rarefaction ou une disparition des anses capillaires
- des modifications morphologiques des anses

V. THERAPEUTIQUES MEDICALES

5.1 Face à l'inflammation, les essais thérapeutiques ont été effectués avec la superoxydismutase (SOD): on a observé une nette réduction des phénomènes inflammatoires entraînant une réduction notable de la phase de restauration.

5.2 L'infection a fait l'objet de traitements préventifs et curatifs:
- la prévention a été obtenue par mise en œuvre de prototypes d'isolement segmentaire: notamment des isolateurs plastiques adaptés aux extrémités des membres
- les traitements curatifs antibiotiques ont été entrepris sur la base des données fournies par l'antibiogramme.

5.3 La douleur est présente à tous les stades de l'évolution, et peut même se manifester après traitement et guérison. Elle est complexe et pose de difficiles problèmes thérapeutiques nécessitant souvent la coopération de spécialistes. Les résultats ont été obtenus par des moyens médicaux, physiques et chirurgicaux. En plus des traitements classiques faisant appel aux anti-inflammatoires, aux analgésiques, aux sédatifs, aux anxiolytiques, aux hypnogènes et aux anti-dépresseurs, on a obtenu les résultats intéressants qui suivent:

- vasodilateurs utilisés localement selon la technique dite de "bloc chirurgical", en particulier pour les douleurs du membre supérieur

- médicaments à impact cortical pour les douleurs fantômes: agents physiques telles que la neurostimulation transcutanée métamérique, libérant des endorphines

- l'excision en cas d'ulcération nécrotique.

VI. THERAPEUTIQUES CHIRURGICALES

Le traitement chirurgical intervient pour les ulcérations profondes et les nécroses potentielles ou constituées. Il est essentiellement à visée conservatrice, mais certains cas exigent l'amputation.

Les résultats obtenus portent sur les indications thérapeutiques et sur les modalités techniques.

6.1 Indications thérapeutiques

Les résultats indiquent que les indications thérapeutiques doivent être posées très rapidement en vue d'une intervention unique si possible, ce qui ne peut être obtenue qu'n disposant d'un faisceau d'informations diagnostiques fourni par le dosimétrie physique, les examens cliniques et paracliniques thérmiques et vasculaires.

6.2 Modalités techniques

Les résultats chirurgicaux ont permis de préciser une stratégie rationnelle, notamment pour les brûlures radiologiques profondes.

D'une part, l'excision doit n'être ni insuffisante ni excessive.

D'autre part, selon l'état favorable ou non des tissus conjonctivo-vasculaire sous-jacents, le recouvrement peut faire appel à des techniques de greffes ou à des lambeaux pédiculés ou libres.

VII. CONCLUSIONS

Les conclusions essentielles que l'on peut tirer des résultats concernant les irradiations partielles accidentelles sont les suivantes:

1. La distribution superficielle ou profonde de l'irradiation est une donnée capitale pour le pronostic et le traitement.

2. Cette information ne peut être obtenue par la seule sémiologie clinique; la dosimétrie physique fournit des données intéressantes mais relatives; la thermographie et la scintigraphie fournissent des éléments complémentaires. Seule une synthèse de l'ensemble des donnnées diagnostiques permet de poser des indications thérapeutiques rationnelles.

3. Les irradiations partielles accidentelles et superficielles guérissent normalement grâce à l'isolement et avec des traitements médicaux.

4. Les irradiations partielles accidentelles profondes ne peuvent actuellement être traitées de façon optimale que par l'association d'isolement, de traitement médicaux et de techniques chirurgicales conservatrices.

5. En cas d'accident impliquant un grand nombre de victimes potentielles, un triage est nécessaire. Dès le premier jour, en l'absence de manifestation clinique, les examens thermographique et scintigraphique permettent d'affirmer ou d'infirmer une surexposition partielle accidentelle.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

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Dr V.MITZ, Hôpital Boucicaut, 78, rue de la Convention, 75015 Paris. FRANCE

V. Publications:

GONGORA R., MAGDELENAT, H. Acute irradiation accidents in France and their symptomatology. Br.J.Radiol., Suppl.19: 4, 1986

GONGORA, R. Irradiations accidentelles. Estratto da: Rivista di Medicina del Lavoro, Ed.Igiene Industriale, Idelson, Napoli, Anno X, Gennaio-Dicembre 1986

GONGORA, R., STROMBI, E., PERDEREAU B., LE GAL, M., TORMENTA, S. Thermographie et irradiation partielle aigüe. Archivio di Scienze del Lavoro, V (1), 1989

Title of the project no.: Projet 2

Head(s) of project:

Scientific staff: H.MAGDELENAT
Dr J.M.COSSET
Dr E.GLUCKMAN
Dr A.LAUGIER

I. Objectives of the project:

Le projet n°2 concernait le diagnostic et le traitement de l'irradiation globale: indicateurs biologiques de l'irradiation globale. En fait, le deuxième projet a porté non seulement sur les indicateurs biologiques, mais également sur les facteurs biologiques et pharmacologiques aidant à la restauration des tissus hématopoiëtiques.

II Objectives for the reporting period

III. Progress achieved:

A. METHODOLOGIE

I. MODALITES EXPERIMENTALES ET CLINIQUES:

Les études ont été effectuées soit in vitro, soit sur le singe "Macacus fascicularis", soit sur des patients irradiés thérapeutiquement ou accidentellement.

II. PROTOCOLES D'IRRADIATION

Les modalités d'irradiation ont été les suivantes:
1. Des protocoles d'irradiation in vitro ont porté sur des lymphocytes humains du sang circulant soumis à l'irradiation unique de 1 à 10 Gy (essentiellement à 4 Gy).

2. Pour les singes: 3 protocoles d'irradiation:
2.1 Irradiation globale unique à 2 Gy
2.2. Irradiation globale de 8 à 10 Gy, étalée sur 4 à 5 semaines et fractionnée à 0,4 Gy par jour.
2.3 Irradiation globale continue de 5 à 8 Gy

3. Pour les patients: 5 protocoles d'irradiation:
3.1 Irradiation globale unique de 10 Gy (avec protection pulmonaire à 8 Gy).
3.2 Irradiation globale de 12 Gy étalée sur 3 jours et fractionnée sur 6 séances.
3.3 Irradiation globale de 13,2 Gy étalée sur 4 jours et hyperfractionnée sur 11 séances.
3.4 Irradiation thoraco-abdominale unique à 1 Gy et à 6 Gy avec protection des poumons et du foie.
3.5 Irradiation localisée à la glande mammaire (60 Gy) et au sternum (45 Gy), étalée sur 6 semaines et fractionnée sur 30 séances.
- et, en cas d'irradiation accidentelle: évaluation de la dose par dosimétrie physique après reconstitution d'accident.

REMARQUE: Pour un certain nombre de patients, le traitement était mixte (chimiothérapie + radiothérapie ou radiothérapie + chimiothérapie).

III. METHODES D'INVESTIGATION

Les méthodes d'investigation utilisées ont fait appel à la clinique, à l'hématologie, à la biochimie, à la cytogénétique, à la biologie moléculaire, à la physiopathologie et à la thérapeutique.

1. Clinique:

1.1 Etude du syndrome GVH (réaction du greffon contre l'hôte), aigu ou chronique.

1.2. Etude de la toxicité à court terme (maladie reino-occlusive du foie, troubles fonctionnels respiratoires, inhibition des sécrétions salivaires et lacrymales).

1.3. Etude de la toxicité à long terme (échocardiogramme, études endocriniennes, troubles de croissance).

2. Hématologie

2.1 Etude de la dépression hématologique immédiate post-radique (granulocytes et lymphocytes et typage HLA)

2.2 Etude de la restauration hématologique

3. Biochimie

3.1 Etude des indicateurs hormonaux de l'irradiation (cortisol plasmatique, ACTH, adrénaline et nosadrénaline, hormone thyroïdienne et TSH).

3.2 Etude des indicateurs métaboliques: gaz expirés: éthane - penthane.

4. Cytogénétique

4.1 Etude des aberrations chromosomiques instables: dicentriques et acentriques

4.2 Etude de la distribution statistique (Poissonnienne), liée à l'hétérogénéité de l'irradiation

4.3 Etude des variations de la radiosensibilité chromosomique

4.4 Etude du chimérisme hématopoiétique après allogreffe

5. Biologie moléculaire

5.1 Etude des ruptures d'ADN et des systèmes de réparation dans les lymphocytes humains

5.2 Etude de la capacité de réparation des lésions d'ADN

6. Physiopathologie

6.1 Etude de la myélofibrose (PDGF, TGFß, TGFα)

6.2 Rôle des facteurs fibrosants plaquettaires

6.3 Etude du stroma médullaire et de la myélofibrose

7. Thérapeutique

7.1 Traitement des insuffisances médullaires secondaires à une allograffe par le GMCSF

7.2 Traitement des aplasies médullaires par transplantation à partir de sang de cordon ombilical.

B. RESULTATS

Ils portent sur certains effets pathologiques post-radiques, sur les indicateurs biologiques des effets de l'irradiation et sur la restauration hématopoiétique.

I. EFFETS PATHOLOGIQUES POST-RADIQUES

1 Pathologie hépatique: mise en évidence de l'induction de maladie veino-occlusive du foie chez des patients soumis à des traitements chimio-radiothérapiques.

2 Pathologie pulmonaire: modifications des épreuves fonctionnelles respiratoires chez des patients soumis à une irradiation pulmonaire de 8 Gy.

3 Pathologie des glandes salivaires et lacrymales: constatation d'une inhibition des sécrétions salivaires et lacrymales chez des malades ayant subi une allogreffe après irradiation.

4 Effets cytogénétiques:

- irradiation partielle: une irradiation partielle étalée et fractionnée sur 6 semaines, mammaire et sternale, de l'ordre de 55 Gy, correspond pour les aberrations chromosomiques lymphocytaires à une irradiation globale unique de 2 Gy.
- l'étude, sur un nombre de cas limités chez l'homme, de la distribution poisonnienne des altérations chromosomiques lymphocytaires, n'a permis qu'une appréciation qualitative de l'hétérogénéité de dstribution spatiale des doses.

- l'étude de la radiosensibilité chromosomique lymphocytaire par irradiation in vitro surajoutée à une irradiation thérapeutique in vivo, a montré:

- des modifications de la réparation des aberrations dicentriques et acentriques, ainsi
- qu'une altération de la capacité de réparation des lésions de l'ADN.

5. Fibrose

Parmi les facteurs fibrosants plaquettaires (EGFR ou TGFα, PDGF, TGFß), le rôle du facteur TGFß est apparu prépondérant dans un modèle de fibrose médullaire.

II. INDICATEURS BIOLOGIQUES DES EFFETS DE L'IRRADIATION

1 Indicateurs hématologiques:

1.1 Le pic granulocytaire constant entre 4 et 8h après une irradiation globale unique de 10 Gy, n'est plus significatif au-dessous de 2 Gy.

1.2 La chute lymphocytaire après une irradiation globale unique de 10 Gy a été confirmée égale à 50% en 8h et à 75% en 24h.

1.3 Le typage HLA a été impossible à réaliser dans un cas sur cinq après 18h et dans 2 cas sur 5 après 24h, dans le cas d'une irradiation globale unique de 10 Gy.

2 Indicateurs hormonaux

2.1 Après une irradiation globale unique de 10 Gy, on a constaté pour le cortisol et l'ACTH, dans les 4 premières heures, un pic constant compris entre 4,5 pour le cortisol et 6,5 pour l'ACTH.
Les taux d'adrénaline et d'hormones thyroïdiennes n'ont montré aucune modification significative

3. Indicateurs enzymatiques

Après irradiation globale, un pic d'amylasémie est constant. Il est dose-dependant. Le seuil de réponse se situe entre 0,5 et 1 Gy et on a montré qu'il n'existait pas, si les glandes salivaires n'étaient pas irradiées.

4. Indicateurs métaboliques

Des différentes techniques ont rendu impossible l'obtention de résultats concluants chez l'homme de la mesure de l'éthane et du penthane expirés.

III. RESTAURATION HEMATOPOIETIQUE

1. Les premières transplantations à partir de sang de cordon ombilical permettent d'entretenir la possibilité de succès dans des situations qui ne seraient pas strictement HLA identique.

2. L'utilisation de facteurs de croissance GMCSF a permis une reconstitution médullaire totale chez des malades atteints d'une insuffisance médullaire secondaire à une allogreffe dans un pourcentage important de cas.

DISCUSSION

I. EFFETS PATHOLOGIQUES POST-RADIQUES

1. Pathologie hépatique: les maladies veino-occlusives du foie chez les patients traités par chimio-radiothérapie, semblent dues à la chimiothérapie ou à l'existence d'une sérologie positive à cytomégalovirus avant greffe.

2. Pathologie pulmonaire: les modifications des épreuves fonctionnelles respiratoires apparaissent majorées en cas de réaction du greffon contre l'hôte et en cas de pneumonie infectieuse, en particulier à cytomégalovirus.

3. Pathologies des glandes salivaires et lacrymales: on a pu differencier les troubles salivaires dûs à l'irradiation des lésions liées à une réaction GVH chronique.

4. Effets cytogénétiques

- Il convient d'être prudent dans l'interprétation des aberrations chromosomiques après irradiation accidentelle du fait de distribution spatio-temporelle différente de doses qu'une analyse statistique poissonnienne peut dans certains cas, aider à élucider.
- L'irradiation lymphocytaire surajoutée in vitro, après irradiation in vivo, permet de mettre en évidence des modifications de la radiosensibilité chromosomique et de la capacité de réparation des lésions de l'ADN.

II. INDICATEURS BIOLOGIQUES

1. Indicateurs hématologiques

En cas d'irradiation accidentelle, il apparaît capital d'effectuer des examens hématologiques toutes les 2h dès le 1er jour en de cerner le pic granulocytaire et la chute lymphocytaire. Par ailleurs, le typage HLA doit, dans toute la mesure du possible être effectué dans les premières douze heures.

2. Indicateurs hormonaux

Parmi les indicateurs hormonaux, les pics d'ACTH et le cortisol sont caractéristiques, à condition d'effectuer le dosage dans les 4 premières heures de l'irradiation.

3. Indicateurs enzymatiques

L'amylasémie à 24h apparaît comme un indicateur sensible avec un seuil inférieur à 1 Gy à condition que les glandes salivaires aient été irradiées.

III. RESTAURATION HEMATOLOGIQUE

1. En cas d'insuffisances médullaires secondaires à des allogreffes, qu'elles soient liées à une GVH aigüe ou à une infection à CMV, l'utilisation de GMCSF permet dans un fort pourcentage de cas, une reconstitution médullaire totale.

2. Dans des situations qui ne seraient pas strictement HLA identique, des espoirs de succès paraissent permis en utilisant des transplantations à partir du sang de cordon ombilical.

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V. Publications:

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-C-071-B

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Title of the research contract:

Late somatic effects of radiation in mammals.

List of projects:

1. Late effects of an in utero irradiation on the central nervous system.
2. Early and late radiation damage to the hemopoietic and immune system of new-born animals.
3. Effect of fractionation of gamma rays and neutrons on cancer induction and promotion in mouse liver.
4. The effect of age on tumour induction by radiation alone or combined with a chemical carcinogen.

Title of the project no.: 1

Late effects of an in utero irradiation on the central nervous system

Head(s) of project:

M. Janowski, G.B. Gerber, H. Reyners

Scientific staff:

E. Gianfelici de Reyners, L. Regniers

I. Objectives of the project:

Our aim is to reveal and study the mechanisms of fetal radiosensitivity of the central nervous system using a combined morphological and biochemical approach. We also intend to determine the dependence on dose and radiation quality using a spectrum of irradiation protocols.

II. Objectives for the reporting period:

A. Morphological assays :

- For X-ray doses of 1 to 15 cGy and 600 keV and 2.5 MeV neutron doses of 1 to 15 cGy administered at different ages, changes in body and brain weight, and in ultrastuctural brain parameters;
- Comparison between low dose rates of gamma rays and high dose rates of X-rays administered at day 15 post conception;
- Relative biological effectiveness of X-rays versus neutrons.

B. Biochemical studies :

- For X-ray doses of 0.5 to 2 Gy at different fetal stages, changes in body and brain weight, biogenic amines, amino acids, and receptors.

Progress achieved:

The observations made at Hiroshima and Nagasaki have drawn attention to the risk of mental retardation after exposure in utero during the early period of pregnancy. The data do not allow to decide whether a threshold dose exists for this effect. Indeed, a linear non-threshold dose effect relationship for mental retardation, if demonstrated, would have important consequences for radiation protection regulations and practices.

It is unlikely that human data will become available which can resolve the problem; therefore, one must search for animal models to study evolution and mechanisms of radiation-induced changes in utero. Obviously, no animal model, except perhaps primates, can give a fully satisfactory answer although they can give useful indications. The present studies concentrate on two important aspects, morphological and biochemical changes in the adult rat after exposure in utero at different times of pregnancy.

I. Morphological Studies.

Introduction.

Little attention has been devoted up to now to the hazards of very low doses of irradiation (<20 cGy) on the development of CNS. However, in close relationship with the present exponential development in microelectronics and in quantitative image analysis, numerous developmental abnormalities can now be detected at much lower dose levels than foreseeable just a few years ago.

It is also our conviction that the relatively important anatomical alterations which were found by such methods and are described below, must be the consequences of numerous transient and/or chronical changes occurring at the deeper ultrastructural, macromolecular and molecular levels; the biochemical part of this report deals in considerable detail with a selection of these latter alterations. We assume that the present studies will provide unvaluable clues leading towards the discovery in the near future of more subtle abnormalities present even after lower doses of irradiation (<1 cGy ?) and eventually close enough to the exposure range found in everyday life.

Material and methods.

The methods involved in this research largely belong to general morphology. However, a few peculiar aspects must be pointed out here:

1. The use of standardized animal litters. The major goal of the present investigations is to reveal brain alterations after the lowest possible doses of irradiation. However, biological variability is in general so high that very faint effects could get lost. In an attempt to control at least a part of the variations, only animals born in litters of 8 to 10 pups were considered. Moreover, these selected litters were reduced to 8 pubs one day after birth. We are convinced that such protocole enabled us to detect tissular involutions at the very low dose levels described below.

2. All irradiations are performed on day 15 post conception (pc). This date corresponds to a

particularly active period of cerebral organogenesis, and consequently also to a stage of major radiosensitivity.

3. Quantitative image analysis. Among the various brain parameters considered as indicators of abnormalities, the most sensitive are those integrating a large number of tissue components; namely, measurements of tissular areas or volumes. The quantitation of these parameters was performed using a digitizing graphic tablet interfaced to a PC. Such data proved to be more sensitive to radiation injury than the brain weight since they pinpoint selected tissular areas which are specifically proliferating on day 15 pc and are consequently exposed to a major risk.

Results and Discussion.

1. X-ray exposure.

a) Major changes in body weight and brain weight.

Body weight and brain weight still range among the best available indicators of general brain damage induced during its organogenesis. Hamilton et al. (1989) used this simple measurement but were only able to recognize a significant decrease in brain weight after doses >= 80 cGy gamma rays given at day 28 pc in the beagle. In the Wistar R/cnb rat used in Mol, X-irradiation given at day 15 pc causes much more severe effects. A tendency to brain atrophy can be noted after doses as low as 5 cGy. Actually, a significant microcephaly is found after 10 cGy if one exclusively uses animals born in standardized litters of 8 +/-1 pups. Such situation will even deteriorate using 600 KeV neutrons as shown in Table I.

b) Minor changes in the glial distribution of the cerebral cortex.

In the adult rat CNS, the glial tissue is the cellular component at major risk after an irradiation. It was indeed shown by us in the recent past (1982,1986a,1989), that a total eradication of a specific glial cell type characterizes the first effects of an irradiation. This radiosensitive glial cell displays many characteristics of immature cells and could be considered as a residual multipotential embryonary cell still persisting in the adult brain. A long time after irradiation, these cells come back but other alterations develop: the so-called late effects of ionizing radiations. These degenerative changes consist in the development of fibrosis in astroglia, a depletion of oligodendroglia but also an hypertrophy of certain capillary blood vessels (telangiectasia).

Since glial changes were prominent after irradiation of the adult brain, it was initially thought that a modified glial radiobehaviour could also be recognized after prenatal irradiation. P. Rakic work in the eighties highlighted the primordial role of a prenatal glial component, the radial glia, during the setup of the various brain substructures and in particular, of the cerebral cortex. According to his views, radial glia multiplies prior to the cortical development, in order to provide a scaffolding along which the different neuronal cell types will migrate and ultimately settle up. It could thus be assumed that the effects of radiation injury bearing on radial glia would become largely amplified due to this leading architectonic role.

However, a study of 15 month old adult rats which had undergone prenatal irradiation with 45 cGy at day 15 pc does not reveal major variations of the glial populations (Reyners, in Kriegel et al., 1986b). In fact, the glial cell densities significantly increased but cell sizes were significantly smaller than in the controls. Computation revealed that the total cell amounts were actually not modified. On the other hand and possibly in relation with this cellular atrophy, a grey and white matter atrophy were clearly evident.

c) Decrease of the thickness of grey and white matter.

Grey matter: the thickness of the cerebral cortex has significantly decreased by 16.7% in the 15 month old brain after in utero irradiation with 45 cGy at day 15 pc. However, here also the total amount of nerve cells has not decreased although cell size is significantly diminished. According to Schneider and Norton (1981) and Konermann (1987), the neural cell processes have reduced and degenerated.

Atrophy of the cingulum: The latter observations also valuably explain why the cingulum bundle (a corpus callosum subsidiary which is made of numerous myelinated axons arising from the neurons of the surrounding cerebral cortex) is deeply diminished (up to 35.2%) after 45 cGy X-irradiation at day 15 pc (Table II). Due to the sensitivity of this criterion, it was attempted to evaluate this structure as exactly as possible.

It was found that its cross-sectional area normally decreases nearly linearly along the antero-posterior axis of the brain. In such conditions, it was decided to measure cingulum areas at various levels along the brain axis (which were estimated thanks to an examination of the complex morphology of the subjacent hippocampus). On the resulting plot, the area located below the line joining all the measurements and between 2 arbitrarily chosen reference positions (anterior and posterior) was called the cingulum volume. This parameter was found to represent the best morphological indicator of radiation injury actually known (Table III); this is also valid for all the other irradiation shedules analysed in this report. As far as X irradiation is concerned, significant atrophy of the cingulum was observed in 3 month old female rats after as low as 10 cGy X-rays at day 15 pc. Of course, larger effects are observed after neutron irradiation.

2. Neutron exposure (600 KeV and 2.5 MeV).

a) Major changes in brain weight.

A most comprehensive study deals with the 600 KeV neutrons. The data are given in table I.

TABLE I. BRAIN WEIGHTS after 600Kev NEUTRON IRRADIATION in UTERO at day 15 pc. (Mean weights in mg +/- s.e.m, *: significantly different from controls; n= number of rats per experimental point.)

AGE	DOSE (cGy)					
	0	1	2.5	5	10	15
3mo	1369±8 (n=30)	1319±14 (n=15)	1307±8 (n=20)	1292±8 (n=18)	1191±8* (n=14)	1072±8* (n=11)
15mo	1519±11 (n=15)	1498±18 (n=10)	1469±11* (n=14)	1421±19* (n=9)	1303±13* (n=10)	
24mo	1543±9 (n=23)	1541±10 (n=15)	1513±10* (n=18)	1461±9* (n=16)	1346±20* (n=10)	1243±21* (n=9)

Brain weight is an excellent indicator of CNS alteration. However, some of the data are to be considered with caution: in 3 month old rats, a significant difference ($p<0.005$, not indicated) was detected between the 30 controls and the 15 brains treated with 1 cGy. It appears that uncontrolled factors have certainly played an important role in such observations. Indeed, NO significant difference in brain weight was found between the groups treated with 1, 2.5 and as much as 5 cGy. The significant difference which exists between the 5 and 10 cGy brains is certainly more meaningful.

In rats of 15 and 24 months of age, the difference between the controls and the 1 cGy brains is clearly unsignificant. In these conditions, the significant difference which appears between the previous 2 groups and the 2.5 cGy rats, could really be considered as real. It also appears that the effects are more severe in the old adults than in the younger 3 month old animals. A similar trend was observed for the X-irradiated cingulum volume and so is in favour of the latter observation (Table II).

b) Severe atrophy of the cingulum.

Neutrons provoke an atrophy of the cingular white matter as shown in Table III.

TABLE II. The effect of TIME after 45 cGy of in utero X-irradiation (at day 15 pc) on the CINGULUM VOLUME. (Figures give the percentage of atrophy with respect to matching controls; n=5; the effect of TREATMENT increases significantly with AGE (p interact. <0.02) in a 2-way ANOVA).

AGE	1mo	3mo	6mo	15mo	24mo
Effect	-20.6%	-21.8%	-34.1%	-24.3%	-35.2%

TABLE III. CINGULUM VOLUME of 3mo rats after IRRADIATION in UTERO at day 15 pc. (Mean volumes in arbitrary units +/- s.e.m; *: $P<0.05$; TMT :treatment; NTN :neutron)

TMT	DOSES (cGy)							
	0	1	2.5	5	10	15	20	100
NTN 2.5 Mev	40.9±1.8 (n=5)			39.5±2.1 (n=5)	27.9±3.7* (n=5)	19.5±3.5* (n=5)		
NTN 0.6 MeV	44.4±1.5! (n=20)	42.5±1.8 (n=15)	40.1±1.8 (n=16)	36.8±1.8* (n=15)	12.4±3.6* (n=7)			
X-ray 250 Kvp	44.7±1.0! (n=4)			41.5±3.4 (n=4)	33.7±1.8* (n=4)	30.4±3.4* (n=4)	27.3±1.5* (n=4)	
GAMMA Co60	39.6±2 (n=8)						26.7±2.3* (n=8)	5±4* (n=7)

3. Gamma ray exposure versus X-rays.

Low dose rates of gamma ray exposures were utilized in order to compare with the effects of high dose rates (of X-rays) at day 15 pc. As shown in Table III, there is no difference between these 2 kinds of irradiation (for 20 cGy exposures).

Other studies now in progress will bear on the effect of very low dose rates of gamma rays administered during the last week of pregnancy or the whole of it: they will deal with the problems of the possible threshold after low dose exposure.

4. Relative Biological Efficiency (RBE).

RBE values can be derived from table III for the cingulum atrophy. The values were computed with respect to X-rays and are relatively modest for this endpoint since they range from 2.78 for the 600 KeV neutrons to a low 1.57 for the less efficient 2.5 MeV ones.

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II. Biochemical Studies.

Introduction:

The study aimed to elucidate the mechanism of action of radiation on the developing brain and its consequences by using an experimental model which could give information on brain activity in the adult. For this purpose, we have investigated the level of biogenic amines and their receptors in different structures of the adult rat brain at different ages after exposure of the pregnant mother.

Material and Methods

Rats were **exposed** at day 10, 12 or 15 of pregnancy to 0.5, 1 or 2 Gy of X rays (200 KVvp, filter 1mm Cu, about 1 Gy/min). Timing of pregnancy was ascertained from the vaginal plug after overnight mating.

At different ages, the offspring was sacrificed, the brain was dissected and the following structures were isolated by dissection: cortex, hippocampus, striatum, thalamus, hypothalamus, cerebellum, medulla. The tissues were frozen until assay and pooled where needed.

For the determination of **Biogenic amines and amino acids**, the tissues were homogenized in perchloric acid to which a small amount of ascorbic acid had been added, and the supernatant was used for column chromatography after neutralisation. Biogenic amines were determined by HPLC followed by electrochemical detection.

Amino acids and related biogenic amines were reacted with o-phthalaldehyde, separated by HPLC chromatography and determined by fluorimetry. The most important and most consistently found substances were: epinephrine, norepinephrine, dopamine, dopa, serotonin, glutamic acid, glutamin, GABA, aspartic acid and taurine. However, some data are also available for DOPAC, 5-hydroxyindolacetic acid, glycine, histidine, thyrosine, ethanolamine and some unidentified compounds.

For the determination of **receptors**, the membrane fraction was isolated by centrifugation and incubated with a range of concentrations of radioactive ligands in the presence and absence of competitors in order to distinguish between specific and non-specific binding. The data were evaluted for the maximal binding capacity (Bmax in femtomoles/mg tissue) and the dissociation constant (Kd, nanomoles) by non-linear analysis of the Michaelis-Menten type hyperbolic function and, after linearisation, by the Scatchard procedure. Usually, the direct non-linear regression yielded more reliable values. It should be pointed out that many receptors can be determined only with a relatively high error (15-20%) since the results are obtained as the difference between total and non-specific binding where the latter often can be as much as 50% of the total binding of a lignand. Autoradiography of the receptors was performed on tissue blocks sectioned in 20 μ sections. The sections were incubated with the radioactive ligand at 25°C for 60 minutesin the presence and absence of the specific competitor, briefly washed 4 times with Tris buffer and exposed to an ^{3}H LKB film for 20 days at -80°C.

The following receptors were determined with the radioactive ligand and the competitor shown in parenthesis: S2-serotonin receptors (^{3}H-ketanserin, methysergide); cholinergic {muscarinic} receptors (^{3}H-quinuclidinyl benzilate, atropin or pirenzepine); H1-histamin receptors) (^{3}H-Pyrilamine, triprolidine); opiate receptors (^{3}H-fentanyl, dextromoramide); benzodiazepine receptors (^{3}H-flunitrazepam, clonazepam); D2-dopamin (^{3}H-spiperone, domperidone).

Results

Data on different receptors are shown in the following tables IV. It should be noted that data from only one brain structure is shown, ie, that with the highest content of receptor. Data on other structures, where obtained, are summarized in the legend of the tables. Note statistically significant differences (p 0.05) are indicated as *.

Benzodiazepine Receptors in Cortex
(percent of controls)

(with ^{3}H-flunitrazepam and clonazepam)

Age	B max	Kd
Irrad. 10 days		
1 month old	89	71*
3 months old	102	60*
6 months old	83	78*
Irrad. 12 days		
1 month old	101	78*
3 months old	97	77*
6 months old	91	82
Irrad. 15 days		
1 month old	91	58*
3 months old	97	78*
6 months old	98	87

Note: Both Kd and B max diminish from 3 to 6 months in controls. No changes found in cerebellum after irradiation or with age

D2-Dopamin Receptors in Striatum
(percent of controls)

(with ^{3}H-spiperone and domperidone)

Age	B max	Kd
Irrad. 10 days		
1 month old	110	111
3 months old	108	92
Irrad. 12 days		
1 month old	118	92
3 months old	113	100
Irrad. 15 days		
1 month old	111	78*
3 months old	121	115

Note: no significant change with age is observed

S2-Serotonin Receptors in Cortex
(percent of controls)

(with ^{3}H-ketanserin and methysergide)

Age	B max	Kd
Irrad. 10 days		
20 days old	106	144*
1 month old	102	99
2 months old	92	168*
Irrad. 12 days		
20 days old	105	58*
1 month old	104	61*
2 months old	90	63*
Irrad. 15 days		
20 days old	90	78
1 month old	121	86
2 months old	134*	146*

Note: Both Kd and B max increase slightly with age.

S2-Serotonin Receptors in Striatum
(percent of controls)

(with ^{3}H-ketanserin and methysergide)

Age	B max	Kd
Irrad. 10 days		
20 days old	71*	72*
1 month old	112	95
2 months old	116	195*
Irrad. 12 days		
20 days old	98	91
1 month old	112	81
2 months old	116	148*
Irrad. 15 days		
20 days old	84	70*
1 month old	80	76*
2 months old	74*	180*

Note: Both Kd and B max do not change much with age

Opiate Receptors in Cortex
(in percent of controls)

(with ^{3}H-Fentanyl and dextromoramide)

Age	B max	Kd
Irrad. 10 days		
3 months old	151*	153*
6 months old	152*	125*
Irrad. 15 days		
3 months old	98	141*
6 months old	128*	74*

Note: no significant changes with age (3/6 months). In the hippocampus a slight reduction of both B max and Kd after 3 and 6 months.

H1-Histamine Receptors
(in percent of controls)
(with ^{3}H-Pyrilamine and tripolidine)

Age	B max	Kd
Irrad. 10 days		
1 month old	126	110
3 months old	105	62*
6 months old	102	78*
Irrad. 12 days		
1 month old	103	95
3 months old	121	89
6 months old	103	70*
Irrad. 15 days		
1 month old	94	82
3 months old	68*	78*
6 months old	138*	136*

Note: Both Kd and B max increase with age in controls. No significant changes found in hippocampus after irradiation or with age

Table V Changes in weight of the total animal, total brain and the striatum after exposure in utero to 1 Gy at different times of pregnancy

	Animal weight g		Brain weight mg		Striatum weight mg	
	1 month	3 months	1 month	3 months	1 month	3 months
Control	140	245	1385	1772	59	63
day 10	143	212	1270	1612	53	63
day 12	126	210	1073	1326	40	51
day 15	137	233	1002	1354	36	50

Figure VI Autoradiographic display of specific and nonspecific opiate receptors in normal and irradiated brain at an age of 3 months. No significant differences are observed. Similar observations have been made on diazepine receptors.

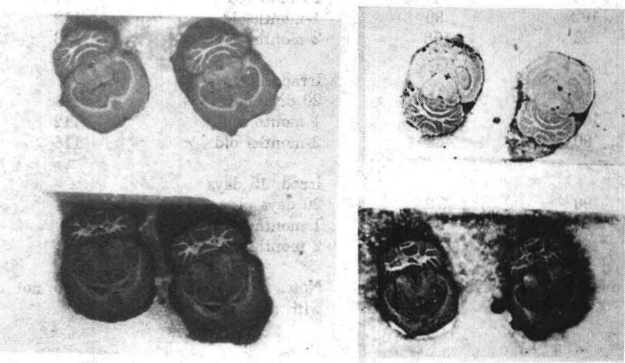
Opiate Receptors

Control

Total Specific

Irradiated

Total Specific



Data on total brain weight, animal weight, and the weight of the striatum are shown in table V. Similar observations were made on other brain structures with hippocampus showing the most marked alterations after radiation.
The data demonstrate that most receptors display an increase rather than a decrease in activity per g tissue after an in utero exposure to 1 Gy, and, for the receptors so far studied by autoradiography, show no marked change in distribution. It should be noted that the error of the determination of some receptors is relatively large (15-20%) since the data are obtained as the difference between total and nonspecific binding where non-specific binding can represent as much as 50% of total ligand bound.

Data on **biogenic amines and amino acids** in different brain areas from rats exposed at 10, 12 or 15 days of pregnancy to 1 Gy at an age of 1 month are shown in the table VII. Data, not shown, have been obtained also for 18 days (2 experiments), 1 months (2 experiments), 2 months, 3 months (2 experiments), 4 months and 6 months after 1 Gy and for 1 months and 3 months after 1.5 Gy. Marked and reproducible changes were found in several neurotransmitters but again these reflect as often increases as decreases. In a general way however, increases seem to be more frequent indicating a tendency to compensate for cell loss by an increase in neurotransmitter content.

Conclusions

The changes found in receptors or neurotransmitters although significant dfound are not very pronounced and represent increases as well as decreases. However, method is subject to a relatively large error (15-20 %) since results are obtained by subtracting a relatively large amount of non-specific from total binding.

Preliminary autoradiographic data while showing clearly different binding in the various brain structures do not indicate so far an important difference between irradiatied and non-irradiated brain areas except where major tissue defects occur.

Biogenic amines and amino acids (not presented in detail) show often an increase in amount when related to tissue mass or no changes when related to total brain structure.

Table VII. Biogenic amines and amino acids in 1 months old rats exposed to 1 Gy on day 10, 12 or 15 of pregnancy

	Cortex	Hippocampus	Striatum	Thalamus	Hypothalamus	Cerebellum	Medulla
Dopamin							
Day 10	128		79	103	128	99	60
Day 12	78		119	60			24
Day 15	101		141	140	70		52
DOPA							
Day 10	91		71	69			
Day 12	83		101	82			
Day 15	122		107	39			
Norepinephrine							
Day 10	53	92		90	113	106	140
Day 12		75		140		102	89
Day 15	195	135		183	149	106	141
Epinephrine							
Day 10	114	92	81	76	118	43	153
Day 12	73	98	106	103		134	75
Day 15	129		117	189	176	135	142
Aspart.Ac.							
Day 10		155*	96	129	115	119	107
Day 12	191	109	95	117	103	124	96
Day 15	143		78	42	86	113	95
Glutamic Ac.							
Day 10	51	127	84	96	106	113	107
Day 12	187*	90	92	124	87	128*	80
Day 15	169*	105	107	75	125*	114	130*
Glutamine							
Day 10	85	131	109	123	111	86	99
Day 12	117	88	77	77	133*	113	104
Day 15	185*	91	99	35*	71	88	70*
GABA							
Day 10	111	133	79	91	128*	94	112
Day 12	205*	85	84	112	113	103	90
Day 15	255*	105	104	107	96	112	100
Taurine							
Day 10	59*	110	59*	88	106	99	112
Day 12	174*	116	78	102	81	121*	86
Day 15	140*	88	146*	72	64*	91	84

Title of the project no.: 2

Early and late radiation damage to the hematopoietic and immune systems of newborn animals

Head(s) of project:

L. de Saint-Georges

Scientific staff:

G.B. Gerber, R. Hooghe, M. Janowski, L. de Saint-Georges

I. Objectives of the project:

The working hypothesis is that the radiosensitivity of the haemopoietic system (and immune system in particular) is different in adult and in developing animals. Short- and long-term consequences of prenatal and neonatal irradiation are therefore studied.

II. Objectives for the reporting period:

The work under the reported period of investigation aimed to test the ability of the irradiated hemopoietic system of neonate and adult mice of different strains to adapt to an increased demand either immediately after the irradiation or after a recovery period of 14, 30, 90 days following the irradiation. The reticulocyte reaction following hypoxic exposure was used as test system.

III. Progress achieved:

1. CAPACITY OF IRRADIATED HEMOPOIETIC SYSTEM OF THE NEONATE AND ADULT MOUSE TO ADAPT TO AN INCREASED DEMAND

Introduction

Fractionation of a dose results in most instances in less damage compared to a single exposure. There exist however several exceptions to this rule one notably being the greater radiosensibility of infant mice to fractionated X-ray exposure.

Mice exposed at day 6 of life seem incapable of repairing radiation damage as do adults and display a greater mortality when dose is split between day 6 and day 9. Maximal sensitization occurs when the conditioning dose is smaller than the test dose.

Mice surviving the acute phase of the radiation syndrome after fractionated exposure also die more frequently from aplastic anemia and thymic lymphoma during the first half year of life than those exposed to a single dose. The difference in the survival after irradiation disappears in juvenile mice.

Material and methods

a) Protocol of irradiation :

Animals were whole body X rays irradiated under a Philips RT 250 tube at photon energy 250 KV, 15 mA using a 1 mm Cu filter (HVL 1.9 mm). The dose rate was 0.95 Gy and total doses were 3.1 Gy in a single exposure or 3.1 Gy fractionated in 1.1 and 2.0 Gy at a 3 days interval. Doses were monitored with a Farmer Baldwin 2570 dosimeter placed in the middle of the field.

b) Hypoxic stimulation :

Erythropoiesis was stimulated by keeping the mice at low pressure in the device described in figure 1. It consisted in an air-tight cage connected to a rotary pump. Pressure in the cage was maintained constand at 350 mbar by means of a periodically switched electromagnetic valve connected to outside air. Pressure in the cage was measured by means of a STATHAM pressure transducer. The signal was amplified, fed into a comparator and the resulting signal was used to activate the valve via an optocoupler.
A 3 days hypoxic stimulation was experimentally determined to be the optimal time for recording the maximal reticulocyte ccunte.
Water and food were given ad libitum.
Immediately after low pressure stimulation, mice were killed and the percentage of reticulocytes was determined on smears after staining with methylen blue.

c) Experimental animals :

6 days and 3 months old BALB/c and C57BL mice from the CEN, Mol, animal facility were used in the experiments.

Results

Reticulocyte reaction varies profoundly according to the strain of mice considered and only BALB/c mice were giving good reproducible results and were further considered as our experimental model (Tabel I).

Table I

	% reticulocytes for each stimulated period			
	0 days	14 days	30 days	90 days
BALB/c 6 days				
Control	21.90±4.36	5.09±2.37	4.91±1.65	2.61±0.71
3.1 Gy	7.63±3.07	6.86±1.88	2.86±0.88	2.95±0.88
1.1 Gy + 2 Gy	8.29±2.74	6.41±2.03	3.80±0.88	4.14±1.47
BALB/c 3 months				
Control	3.39±1.32	2.31±0.55	2.88±0.76	2.74±2.04
3.1 Gy	0.07±0.09	5.96±1.43	1.71±1.00	0.83±0.43
1.1 Gy + 2 Gy	1.34±2.68	4.94±0.89	3.28±0.64	2.45±0.71
BALB/c 1 month	non exposed non stimulated mice		1.81±0.73	
3 months			1.51±0.28	

Differences in the percentages of reticulocytes released in the circulation are recorded for neonate and adult mice all along the experimental time up to 90 days post irradiation.
Non-irradiated, non-stimulated adult BALB/c mice have a reticulocyte count of 1.81% ± 0.73.

In the non-irradiated hypoxy stimulated mice (Control mice), the reticulocyte count was decreasing from 5.6% (9 days old mice) to reach 90 days after a plateau at 2.61 ± 0.71 %.

In the irradiated stimulated mice, three phases should be considered separately : the acute phase, from 0 to about 14 days after hypoxic stimulation, the "repair" phase, from 14 to 30 days and the chronic phase from 30 to 90 days and over.

In adult mice a typical behaviour with immediate damage followed by a repair phase is seen during the first 30 days.
In neonate mice, irradiation results in an immediate high reticulocyte response that gradually decreases with time after irradition.

As seen after 14 days, in either young or adult mice, a single irradiation has a more long-lasting depressing effect that a fractionated one. In juvenile mice however, the values are higher and recovery appears to have rather place after single exposure whereas an increased reaction is maintained after fractionated dose.
One remarquable feature after day 14 is that in both the neonate and adult mice, the roughly similar parallel evolution of the reticulocye counts for the single and fractionated dose. The main difference between young and adult mice residing essentially in a rather constant shift downward of about 2 % of all the count from irradiated mice.

Discussion

The results demonstrate that reticulocyte reaction and hence hemopoietic marrow behaviour differs profoundsly between adult and neonate mice for the period considered.
In both cases a single irradiation leads to lower reticulocyte counts than a fractionated exposure and compared to the control mice, the depletion is more marked in adult than juvenile mice.
The more significant differences are observed during the first 14 days following irradiation where exposure results in a depletion of the reticulocyte count in adult but in an amplified response in young mice. These results on regeneration of the erythropoietic marrow in neonates could be explained by a preferential stimulation of erythropoiesis in irradiated neonates compared to irradiated adults. Indeed it has been observed earlier that the ratio erythroid to granulocytic type of spleen colonies increases in irradiated neonate mice up to day 30.
Another aspect which play an important role in the difference between adults and neonates response to stimulation is the reaction of the stromal cells needed to maintain normal marrow function, and the incomplete marrow functional characteristics in the young mouse. Indeed in juvenile mice, the stromal cells are still undergoing cellular divisions and are therefore much more radio-sensitive than the quiescent adult ones. An indirect stimulating effect on hemopoietic stem cells via a damage to stromal cells and the resulting marrow disfunction could better explain the opposite erythrocyte reaction between adult and neonates.

It is also known that the marrow blood flow rate is under the sympathetic nerves control. This control was shown active in the rat only two weeks after birth. Bone marrow blood flow, stromal cells, and the integrity of endothelium are important for the release capacity of newly produced blood cells from marrow precursors into the systemic circulation. It is therefore evident than a greater radiosensitivity of neonate stromal system and a still uncontroled marrow blood flow could also explain the early differences observed in the reticulocyte response between juvenile and adult mice by an immediate release of reticulocytes into the marrow circulation through the damaged reticulo-endothelium of the medullar system.

The results presented emphasize the importance of stromal cell consideration in the study of the stem cell radiosensitivity in vivo. Erythrocyte response analysis after hypoxic stimulation is a good model for the study of the functional integrity of the marrow after irradiation.

2. HOST STEM CELL BEHAVIOUR AFTER WHOLE BODY IRRADIATION FOLLOWED BY BONE MARROW GRAFT

Introduction

Hemopoietic pluripotent stem cells first appear in the primitive yolk sac, subsequently reach the embryonic liver and hence migrate to the marrow cavity during a period of several days starting before birth to become exclusively medular a few days after birth.
The bone marrow stromal system constitution is a prerequisite to the appearance of hemopoiesis in the bone cavity.

The current view is that stromal cells and hemopoietic stem cells belong to different cell population with their own specific progenitor cells. However several authors suggest that some stromal cells could be at the origin of the hemopoiesis. Islam (1985), from histopathological evidence of human bone marrow biopsies, suggest that endosteal lining cells could be the true stem cells giving rise to osteoprogenitor cells and to hemopoietic stem cells. The problem is thus far from elucidated.

The present experiment investigated the possibility of awakening some quiescent stem cells remaining after letal whole body irradiation by analysing the leukocyte karyotype of hypoxia stimulated recipient mice at different time after marrow graft from mouse cells with a different karyotype.

Material and methods

Normal C57BL/Cnb (40 chromosomes) and C57BL/Cnb with 38 chromosomes (36+2 acrocentrics) obtained by Robertsonian translocation (Léonard et al.) produced and bred in the CEN-Mol animal facility, were used respectively as recipient and donor mice in the bone graft experiment. At 10 days, 1,2,3,4 and 5 months after the bone marrow graft, 8 mice were stimulated by a 3 day hypoxia at 350 mBar as described in the above part of the report.
Blood was then collected and smears were analysed for the presence of the different karyotypes.

Bone marrow graft :

Mice of the 40 chromosome karyotype were irradiated whole body with 8.5 Gy at a dose rate of 0.95 Gy/min (1 mm Cy filter, HVL 1.9 mm) then injected (i.v.) with 500,000 marrow cells from donor mice with the 38 chromosome karyotype.
Group A, was exposed to hypoxia stimulation while the other, groupe B, not.

Results

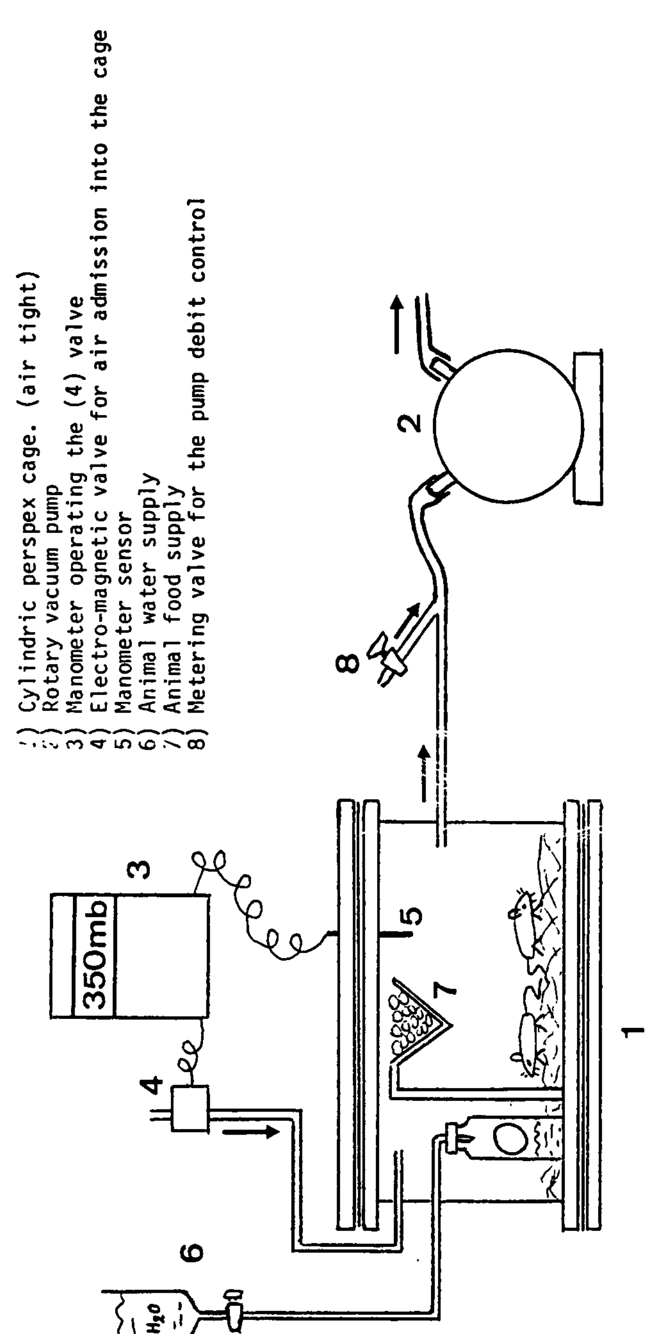
Results are displayed for each group.

Group A :	36+2 chr. (Donor)	40 chr. (Host)	Group B : 36+2 chr. (Donor)	40 chr. (Host)
10 days	-	-	71.4 %	29.26 %
1 month	94.97	5.03	97.51	2.49
2 months	100.0	0.0	96.70	3.30
3 months	98.17	1.83	96.41	3.59
4 months	93.19	6.81	95.77	4.23
5 months	90.07	9.93	93.20	6.80

Control : C57BL/Cnb (38 chr.), 4 months : 100 % 36+2 chr.
C57BL/Cnb (40 chr.), 4 months : 100 % 40 chr.

Discussion

Results shown no significant differences between groups A and B. In the letally irradiated mice, after bone marrow graft, cells with the donor karyotype characteristics (38 chr.) are largely predominant and are responsible of the mouse survival.
The remaining host type cells (40 chr.) survive in important amount till 10 days after irradiation then decrease dramatically thereafter but never completely disappear.
Even after a letal X-irradiation dose, the marrow stem cells pool is not completely destroyed. The host cell karyotype population, after a 10 day period of reduction due to direct radiation damages, is stabilized. This population thereafter does not increase with time and therefore does not suggest any activation of some quiescent host earlier precursors but rather suggest a simple survival and activity of a few of the host stem cells.
Those results determine the basement line of further experiments in which intensive stimulations of the hemopoietic cell pool will be checked for their possible effect on the karyotype ratio between host and donor type. A predominant stimulated reapparition of the host cell karyotype would strongly support the existence of an earlier progenitor than the Hemopoietic Stem Cell as defined by the CFUs assay.



1) Cylindric perspex cage. (air tight)
2) Rotary vacuum pump
3) Manometer operating the (4) valve
4) Electro-magnetic valve for air admission into the cage
5) Manometer sensor
6) Animal water supply
7) Animal food supply
8) Metering valve for the pump debit control
350mb
H_2O
1
2
3
4
5
6
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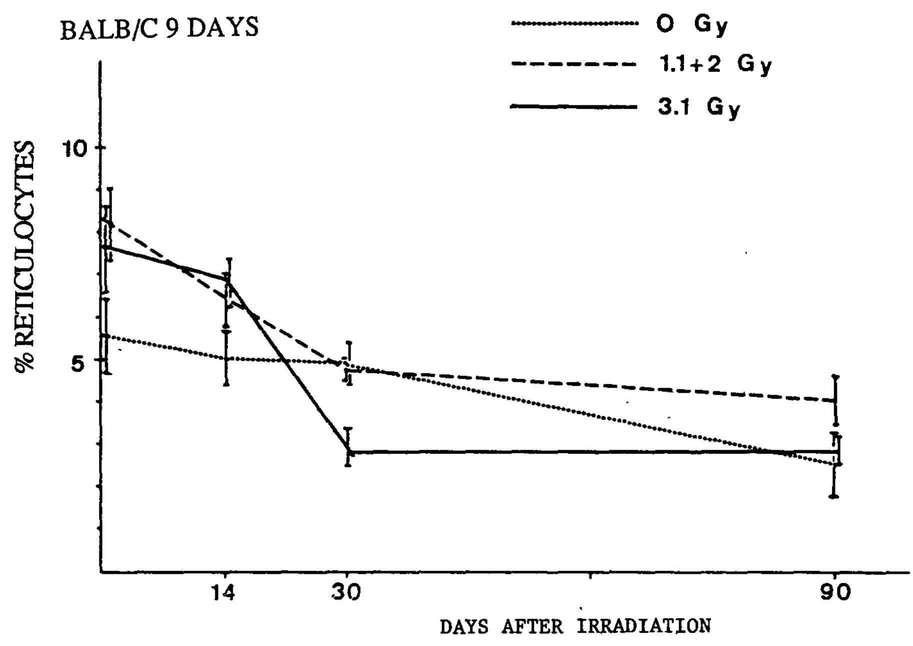
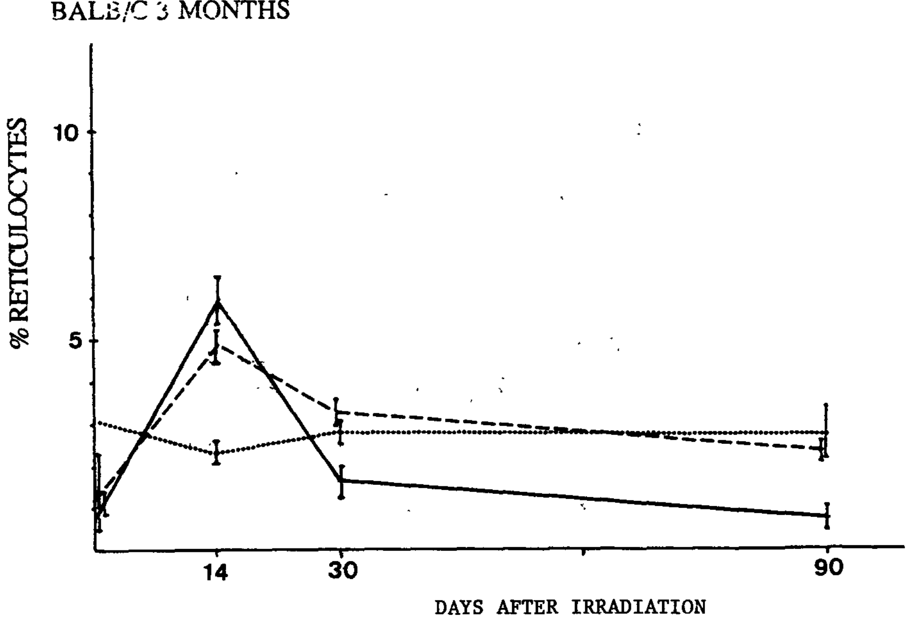
8


BALB/C 9 DAYS
O Gy
1.1+2 Gy
3.1 Gy
% RETICULOCYTES
10
5
14
30
90
DAYS AFTER IRRADIATION


BALB/C 3 MONTHS
% RETICULOCYTES
10
5
14
30
90
DAYS AFTER IRRADIATION

Title of the project no.3: EFFECT OF FRACTIONATION OF GAMMA RAYS AND NEUTRONS ON CANCER INDUCTION AND PROMOTION IN MOUSE LIVER.

Head of project: J.R. Maisin

Scientific staff: J.R. Maisin*, G. Gerber**, M. Janowski***, M. Lambiet-Collier***, G. Mattelin***

* UCL, Brussels, ** CEC, Brussels, *** CEN, Mol.

I. Objectives of the project:

I. to study the effects of X-rays alone or combined with carbon tetrachloride (CCl_4) on induction and promotion of cancer in adult mouse liver ;

II. to carry on the analysis of the final results of the study performed during the period 1980-1984 on the relative effectiveness of a single or fractionated whole body gamma or 50 MeV neutron exposure with respect to survival and cancer induction in C57BL mice.

II Objectives for the reporting period:

1. to determine the lifespan of mice exposed locally on the upper part of the abdomen, with increasing doses of X-rays, treated with a single subcutaneous injection of CCl_4 or exposed to X-irradiation following or preceeding CCl_4 treatment; to perform autopsy and histological analysis of tissue samples of dead mice ;

2. to finalize the analysis of the results of the study performed during the period 1980-1984 on the relative effectiveness of a single or fractionated whole-body gamma or 50 MeV neutron exposure with respect to survival and cancer induction in and C57BL mice.

III. Progress achieved:

I. Effects of X-rays alone or combined with CCl_4 on induction and promotion of cancer in adult mouse liver

1. Methodology

Male C57BL Cnb mice (DL50/30 days 5.75 Gy) 12 weeks old and weighing 25 to 30 g were used throughout.

All mice treated with carbon tetrachloride (CCl_4) (Merck T pur) received a dose of 0.1 ml of a 40% solution of CCl_4 in Miglyol 812 administered subcutaneously in the neck region. All CCl_4 injections were administered between 11 and 12 am.

The conditions of X-irradiation were 250 kV 12.5 mA, dose rate 0.096 Gy/min, 1 mm Cu filter giving a HVL of 1.88 mm Cu. Focus distance 125 cm. During each exposure, the X-ray dose was measured in air using a 2750 A Nuclear Enterprise dosimeter. Mice were fixed on a plastic plate and irradiated in groups of 4 on the upper part of the abdomen. The rest of the body was shielded with 2 plates of lead (5 mm thick). All irradiation were carried out during the morning hours.

The control mice were divided in three groups: normal controls (67 mice), control mice exposed to 0.5 (57 mice), 1 (57 mice), 2 (54 mice), 4 (58 mice) or 6 Gy (57 mice) (Total number of mice: 286 mice) and normal control mice treated with CCl_4 (19 mice).

The treated mice were divided in two groups:
1) X-irradiated mice following CCl_4 treatment. After CCl_4 treatment mice were exposed 69 hours later to 0.5 (60 mice), 2 (56 mice), 4 (57 mice), 6 Gy (57 mice) (Total number of mice: 285 mice)
2) X-irradiated mice preceding CCl_4 treatment. Mice were exposed to 0.5 (56 mice), 1 (58 mice), 2 (113 mice) and 6 Gy (59 mice) and treated three months later with CCl_4 (Total number of mice: 396 mice).

The mice were housed two per cage and kept until death. Autopsies were performed and tissue samples were taken for histological analysis. The following diseases were classified: liver adenoma and carcinoma, liver sarcoma, liver angioma, liver angioma + liver sarcoma, liver adenoma + sarcoma + carcinoma. Thymic lymphoma, myeloid leukaemia, adenocarcinoma of other organs, sarcoma of other organs.
The data were evaluated by the Kaplan-Meier procedure using as criteria cause of death and possible cause of death.

2. Results

No significant differences exist in survival time between the non irradiated mice treated or not with CCl_4, the irradiated mice without CCl_4 treatment and the irradiated mice following or preceding CCl_4 treatment.

The causes of death in our normal control C57BL male mice are in the following decreasing order of frequency: non cancerous late degenerative changes in lung (56.7 %) 25 per cent of the control mice died from any types of leukaemias. The incidence of liver adenoma + adenocarcinoma and sarcoma (in normal control mice) was 11.9 per cent (Table I). In control mice treated with CCl_4 the incidence of adenoma + adenocarcinoma + sarcoma is twice as large as in normal controls (Table II).

Table I. Incidence of liver tumours in control mice and in mice exposed to increasing doses of X radiation

Treat -ment	Number of mice	Liver tumour incidence in %			
		(1) adenoc. + aden.	(2) sarc.	(3) angio.	(4) all malig tum.
contr.	68	10.5	1.4	1.4	11.9
0.5 Gy	57	17.5	1.75	5.3	19.25
1 Gy	57	10.5	1.75	3.5	12.3
2 Gy	54	24	5.5	5.5	29.5
4 Gy	58	27.6	8.6	10.3	36.2 *
6 Gy	57	21	0	7	21

* The Kaplan-Meier estimates show a low significance (0.05 P`0.08) for all malignant liver tumours between normal control and mice exposed to 4 Gy.

(1) adenoma + adenocarcinoma
(2) sarcoma
(3) angioma
(4) all malignant tumours

Table II. Incidence of liver tumours in mice receiving a single injection of carbon tetrachloride before increasing doses of X radiation

Treat -ment	Number of mice	Liver tumour incidence in %			
		(1) adenoc. + aden.	(2) sarc.	(3) angio.	(4) all malig tum.
contr.	68	10.5	1.4	1.4	11.7
contr.+CCl_4	19	26.31	0	0	26.31
CCl_4+0.5 Gy	60	21.6	1.6	1.6	23.3
CCl_4 + 1 Gy	56	17.8	0	5.3	17.8
CCl_4 + 2 Gy	56	19.6	7.1	8.9	26.7
CCl_4 + 4 Gy	57	24.6	0	8.8	24.6
CCl_4 + 6 Gy	56	30.3 *	3.6	10.7	33.9 *

* The Kaplan-Meier estimates show a high significance ($P<0.01$) for adenoma + adenocarcinomasand all malignant liver tumours between normal control and mice treated with CCl_4 + 6 Gy.

(1) adenoma + adenocarcinoma
(2) sarcoma
(3) angioma
(4) all malignant tumours

The incidence of the different types of liver tumours in irradiated mice without CCl_4 treatment, in control mice treated with CCl_4 and in mice treated before or after irradiation with CCl_4 is given in tables II and III.

Table III. Incidence of liver tumours in mice receiving a single injection of carbon tetrachloride after increasing doses of X radiation

Treat -ment	Number of mice	Liver tumour incidence in %			
		(1) adenoc. + aden.	(2) sarc.	(3) angio.	(4) all malig tum.
contr.	68	10.5	1.4	1.4	11.7
contr.+CCl_4	19	26.31	0	0	26.31
0.5 Gy+CCl_4	56	26.8 *	1.8	10.7	28.6 *
1 Gy + CCl_4	58	18.9 *	6.9	5.2	25.8 *
2 Gy + CCl_4	113	19.5 *	5.2	3.5	24.8 *
4 Gy + CCl_4	113	31.8	6.2	6.2	38
6 Gy + CCl_4	59	44.0°*	10.2	10.2	54.2°*

° The Kaplan-Meier estimates show a high significance ($P<0.01$) for adenoma + adenocarcinoma and all malignant liver tumours between normal control and mice treated with 6 Gy + CCl_4.

* The Kaplan-Meier estimates show a high significance ($P<0.01$) for adenoma + adenocarcinoma and all malignant liver tumours between most of the group of mice irradiated before CCl_4 treatment and the groups of mice irradiated with 6 Gy before CCl_4 treatment.

(1) adenoma + adenocarcinoma
(2) sarcoma
(3) angioma
(4) all malignant tumours

When compared to normal controls only the data on adenoma + adenocarcinoma and on all malignant tumours in mice exposed to 6 Gy preceding or following CCl_4 treatment present significant statistical differences ($P<0.01$).

The same observation was made when the data of the groups of mice irradiated before CCl_4 treatment were compared with control X-irradiated mice exposed to the same doses of X-rays.
When the data of the different groups of mice irradiated before CCl_4 treatment were compared to the data of the mice of the same group irradiated with 6 Gy, very significant results were observed for adenoma + carcinoma of the liver and for all malignant liver tumours for most of the group of mice (Table III).

3. Discussion

The experimental data show that administration of CCl_4 three days prior to X-irradiation result after high dose of X-irradiation (6 Gy) in an increase in the number of adenoma + adenocarcinoma + sarcoma though not to the same extent as when CCl_4 was given three months after exposure to X-rays. These results demonstrate that a combined treatment of a promotor given before or after an X-rays exposure seems to increase, after high irradiation doses, the incidence of liver cancers in C57BL mice. The result obtained for groups of mice promoted with CCl_4 three months after irradiation seems to show that no time lag is needed for promotion.

Following CCl_4 treatment liver tissue is severely damaged: from 1/4 to 1/2 of the central region of each lobule is necrotic and eosinophilic. Mitotic figures are scattered at random throughout the intact parenchyma. Abnormalities are rare in mice receiving X-irradiation preceding CCl_4 treatment. There is a marked increase in the number of mitotic abnormalities compared to CCl_4 treated controls. The percentage of abnormal mitotic figures increased with X-rays doses.

4. Conclusions

The administration of CCl_4 three days prior to irradiation results after high doses of X-irradiation (6 Gy) in an increase in the number of adenoma + adenocarcinoma + sarcoma of the liver though not to the same extent as when CCl_4 was given three months after exposure to X-rays. The results obtained for the group of mice promoted with CCl_4 three months after irradiation seems to show that no time lag is needed for promotion

II. Relative effectiveness of a single or fractionated whole-body gamma or 50 MeV neutron exposure with respect to survival and cancer induction

1. Methodology

Male C57BL Cnb(F58) mice, 12 weeks old and weighing 25 to 30 g were used. Gamma irradiations were performed with a ^{137}Cs unit at doses of 0.25, 0.5, 1, 2, 4 or 6 Gy at a dose rate of 0.3 Gy/min. Irradiation was delivered in a single session, in 10 equal doses delivered 24 h apart, or in 8 equal doses 3 h apart.

Neutron irradiation was performed at the UCL-cyclotron of Louvain-la-Neuve. Neutrons were produced bombarding a thick beryllium target with deuterons (d(50)+Be). The indicated doses are the total absorbed doses $D_{n\gamma}$, including the gamma contribution D_γ which was about 7% at the level of the animals. As far as fractionation is concerned, single neutron doses were compared to eight equal fractions 3 h apart.
The mice were housed two per cage and kept until death. An autopsy was performed, and tissue samples were taken for histological analysis. Fewer than 1% of the mice were lost due to decomposition, cannibalism, etc. The following diseases were classified: thymic lymphoma, nonthymic lymphoma, myeloid leukaemia, lung carcinoma, liver carcinoma, thyroid carcinoma, adrenal carcinoma, various sarcomas, thyroid adenoma, adrenal adenoma, spleen adenoma, and noncancerous late degenerative changes in lung and kidney. For statistical purposes the following groups were also pooled: all leukaemias, all carcinomas and sarcomas, all malignant tumors, and all benign tumors.
The data were evaluated by the Kaplan-Meier procedure using criteria cause of death and possible cause of death. Comparison between individual groups was carried out by a modified Wilcoxon procedure according to Hoel and Walburg; an entire set of different doses from one radiation schedule was evaluated by the procedure of Peto using a program kindly supplied by Dr Wahrendorf and translated by us into Basic for use in a Model IV TRS-80 microcomputer. The data were evaluated for trend, heterogenity, and linearity with respect to cause of death and incidental disease. Calculation of the Cox proportional hazard model was carried out using a Basic program given to us by Chmelevsky and Kellerer.

2. Results

Life-shortening

No significant difference exists in survival time of C57BL mice in function of dose between a single gamma or a single neutron exposure. Both gamma fractionation schedules are clearly less effective than a single gamma exposure in reducing survival. On the contrary, a fractionation of

neutrons seems to be slightly although not significantly more effective in reducing life span than a single exposure, whereas neutron exposure of the thorax does not affect life span. Survival time appears to be a linear function of dose in all the experimental groups studied (Fig. 1).

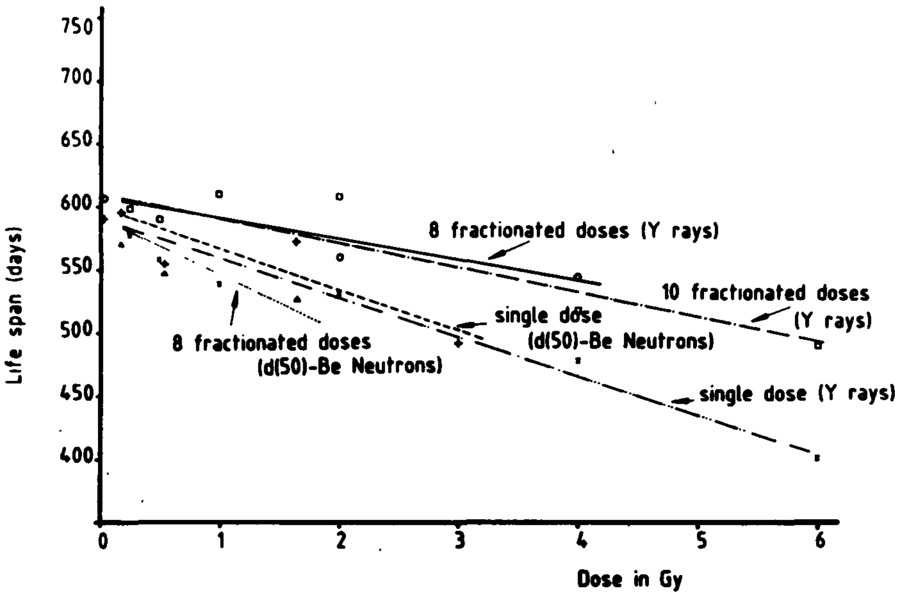


Fig. 1. Dependence of survival time of C57BL mice on dose. Data are shown for single or fractionated (10 fractions 10 days apart and 8 fractions 3 h apart) exposure to gamma rays as well as for single or fractionated (8 fractions 8 h apart) exposure to neutrons.

Life-shortening, calculated by linear weighted regression on dose of the values obtained by the Kaplan-Meier calculation for survival time, amounts to 31.1 ± 2.6 days/Gy for a single exposure, 19.6 ± 2.9 days /Gy for a 10-fraction gamma exposure, and 16.5 ± 3.4 days/Gy for an 8-fraction gamma exposure. For a single neutron exposure, life-shortening is 32.5 ± 5.9 days/Gy; for fractionated neutrons it is 44.8 ± 13.2 days/Gy.

Causes of death

Causes of death in control mice are in the following decreasing order of frequency: noncancerous late degenerative changes in lung and kidney (glomerulosclerosis); 18% of the controls die from any type of leukaemias; and 16% from carcinomas and sarcomas among which liver adenomacarcinoma is most frequent.

The data and the statistical evaluation demonstrate that malignant tumors (particularly leukaemias, including thymoma) as well as noncancerous late degenerative changes in lung are the principal causes of life-shortening after a single high gamma irradiation. A comparison of the crude percentage incidences in function of dose suggests that fractionated exposure, in particular that delivered in 8 fractions 3 h apart, is more efficient in causing malignant tumors and all leukaemias. No difference is noticeable with respect to thymoma. This impression is strengthened by Kaplan-Meier plots: all carcinomas and sarcomas appear to occur somewhat earlier after 1 Gy at a schedule of 8 fractions than after 10 fractions or a single exposure. The same fractionation schedule also is more efficient in causing leukaemias after a dose of 2 Gy which, given as a single dose or in 10 fractions, does not cause earlier leukaemias compared to the other groups (Fig. 2).

Similar observations also pertain to all malignant tumors after 2 Gy. At a dose of 4 Gy, the fractions are less rather than more efficient in causing leukaemias or all malignant tumors than 10 fractions or a single exposure.

Following a single neutron exposure, the crude percentages of leukaemias and thymoma appear to increase somewhat more rapidly with dose than after gamma exposure. There is no clear difference discernable in crude incidences between single and fractionated neutron exposure for all malignant tumors. Because a much larger effect of neutrons had been expected when the experiment was designed, the doses have unfortunately not been chosen in such a way that neutron and gamma exposure could be compared directly for all individual doses. The Kaplan-Meier presentations indicate, however, that after fractionated neutron exposure all malignant tumors occur earlier after a single 1.65 Gy neutron exposure and perhaps still earlier after a fractionated one than after a single 2 Gy gamma exposure. The differences with respect to all leukaemias are less marked for single exposure of neutrons and gamma rays, but fractionated neutrons appear to result in an earlier appearance of leukaemias than a single neutron exposure. Exposure of the thorax only to low doses of neutrons causes a significant increase in leukaemias, all carcinomas and sarcomas, and all malignant tumors, although it does not affect survival time.

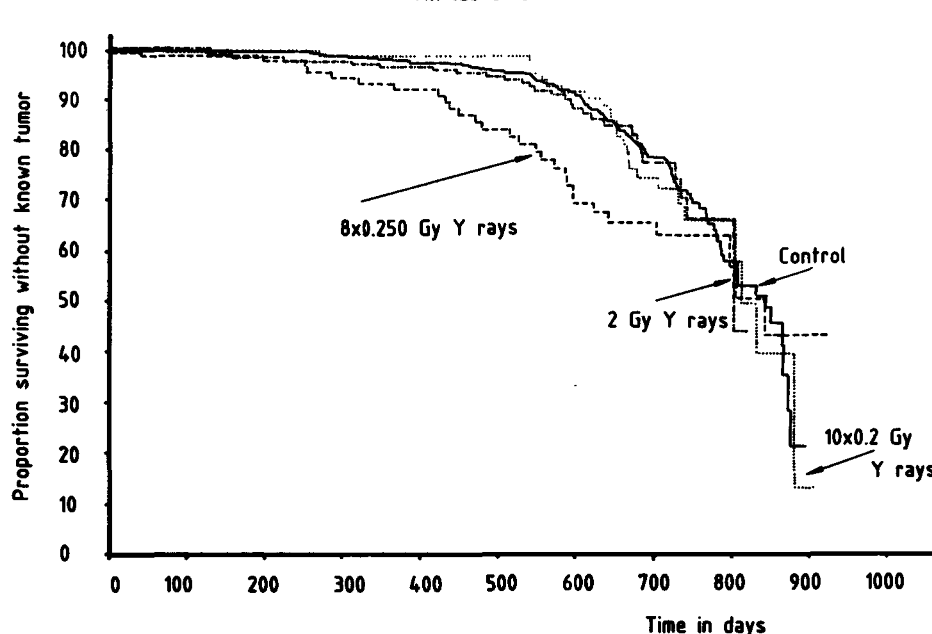


Fig. 2. Probability of surviving without any leukaemia in C57BL mice following a 2 Gy gamma ray dose given in a single exposure, in 8 fractions at intervals of 24 h. Controls are also shown.

3. Discussion and conclusions

Single and fractionated gamma exposure

At equal dose, life-shortening in C57BL mice was slightly less pronounced after a fractionated gamma irradiation than after a single exposure. These observations thus agree with earlier findings on BALB/c mice. Fractionation also appears, however, to result in an earlier and more frequent appearance of tumors after doses in the range of 1-2 Gy (Kaplan-Meier plots) although this difference is not statistically significant. This also confirms the earlier observations on BALB/c mice, a strain which has a much higher spontaneous incidence of tumors than the C57BL and in which

fractionation was also found to be more effective in causing tumors except for thymic lymphoma. Since the average life span is longer after fractionation, it appears that earlier death after single exposure occurs from noncancerous late degenerative lesions.
The differences found in the present study between induction of radiation-induced cancer by fractionated and single gamma exposure are small and not yet well enough established from a statistical point of view. They show, nevertheless, that fractionation of gamma rays would not necessarily be less effective than single exposure in the dose range of about 1-2 Gy.

Single and fractionated neutron exposure

Fractionation of d(50)+Be neutrons appears to cause a more marked life-shortening and an earlier appearance of tumors than single neutron exposure although the statistical significance of the results is not established. No statistically significant difference between single and fractionated neutron exposure could however be observed for all carcinomas and sarcomas and for all leukaemias. Information on the effects of fractionation on tumor induction by d(50)+Be neutrons is not available, but some observations have been made on fission neutrons: in general, fractionation either has no effect or provokes an enhancement. Enhancements are particularly noticeable at higher neutron doses.
Local neutron exposure of the thorax had been carried out in the expectation of an increased incidence of lung tumors, but this was not the case.

RBE for fast neutrons

The RBE for life-shortening of C57BL mice for d(50)+Be neutrons compared to gamma rays was not significantly different from unity. This agrees with our previous data on BALB/c mice. The uncertainty concerning the shape of the dose-effect curve for induction of all malignant tumors precludes a calculation of the RBE; a very rough estimate obtained from the proportional hazard parameter at doses between 2 and 6 Gy for gamma rays and between 0.54 and 3 Gy of neutrons suggests an RBE between 1 and 1.5 for single exposure to gamma rays. The RBE of d(50)+Be neutrons for life-shortening appears to be lower than for other end points. D(50)+Be neutrons are, of course, expected to have much lower RBE than fission neutrons; nevertheless, the RBE values in the present study for life-shortening seem surprisingly low.
Neutron irradiation caused somewhat more cancers than gamma irradiation and the RBE for cancer induction might be higher, probably between 2 and 3 in the range of 1 to 3 Gy, although the present data do not allow a more precise assessment.

IV. Other research group(s) collaborating actively on this project (name(s) and address(es):

V. Publications:

1. PUBLICATIONS IN SCIENTIFIC JOURNALS

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Survival and disease incidence in male BALB/c and C57BL mice after single, fractionated gamma or d(50)-Be neutron exposure.
- 20th Annual meeting of the European Society for Radiation Biology, Pisa (Italy), 15-19 September 1986.

J.R. MAISIN, A. WAMBERSIE, G.B. GERBER,
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Life shortening and disease incidence in C57BL mice after single and fractionated gamma and high-energy neutron exposure.
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- 20th Annual meeting of the European Society for Radiation Biology, Pisa (Italy), 15-19 September 1986.

E. GIANFELICI de REYNERS, H. REYNERS and J.R. MAISIN
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Title of the project no.4: THE EFFECT OF AGE ON LIVER TUMOUR INDUCTION BY RADIATION ALONE OR COMBINED WITH A CHEMICAL CARCINOGEN

Head of project: J.R. Maisin

Scientific staff: J.R. Maisin*, L. de Saint Georges**, M. Janowski**, J. Vankerkom**, H. Liskin***, M. Lambiet-Collier**, G. Mattelin**

* UCL, Brussels; ** CEN, Mol; *** BCMN, Mol.

I. Objectives of the project:

I. to study the influence of age on tumour induction in mice by X- and neutron irradiation

II. to study to which extent small dose of X- and neutron irradiation could potentiate the effects of diethylnitrosamine

II. Objectives of the reporting period:

1. to study to which extent small dose of X- and neutron irradiation could potentiate the effects of diethylnitrosamine (DEN) on the induction of liver tumours

2. to study the influence of age on tumour induction in mouse by X- and neutron irradiation

III. Progress achieved:

1. Methodology

Female C57BL/Cnb mice 8 to 10 weeks old were bread with one male of the same age. Pregnant female were housed singly in plastic cages. Checks for delivery were made twice daily.
At an age of 14 or 21 days, the male offspring was given an μi.p. injection of 0, 0.31, 0.62, 1.25 and 5.0 μg of DEN in 0.9% NaCl solution per μg body weight. The mice were fed ad libitum with standard laboratory chow and water. Animals were kept in a temperature controlled room with a 12 hour light-dark cycle.

The conditions of X-irradiation were 250 kV, 15 mA, exposure rate 0.95 Gy/min, 1 mm Cu filter giving a HVL of 1.85 mm Cu. During each exposure the X-ray dose was measured in air using a 2750-A Nuclear Enterprise dosimeter. Mice were exposed in groups of ten in partitioned plastic cages.

The neutron irradiations were performed at the new thick-target Be(d,n)-field at Ed = 6.3 MeV using a distance of about 55 cm for the source and a pyramidial collimator (2.2 x 2.2 cm at the source and 14.1 x 14.1 cm at the sample position). The neutron spectrum extends to a maximum of 10.7 MeV, the average neutron energy being 3.1 MeV. The doses have been determined for each irradiation as usual with a 0.53 cm^3 thimble ionisation chamber operated in continous TE-gas flow by total charge determination using $\langle W \rangle = (31.9 \pm 1.5)$ eV. Small temperature and pressure corrections were applied.

The 5020 mice used for this experiment were distributed in the following 7 groups :

1. mice treated with DEN alone

Treatment at an age of 14 days with 0.31, 0.62, 1.25 and 2.5 μg/g of DEN (320 mice)

2. mice treated with X-rays alone

Treatment at an age of 7 and 21 with a single dose of 0.50, 1 and 3 Gy of X-rays (480 mice)

3. mice treated with neutrons alone

Treatment at an age of 7 and 21 days with a single dose of 0.25, 0.50 and 1 Gy of 3.1 MeV neutrons (480 mice)

4. mice treated with DEN + X-rays

Treatment at an age of 14 days with 0.31, 0.62, 1.25 or 2.5 μg/g of DEN followed by a single dose of 0.5, 1 or 3 Gy of X-rays 7 days later (960 mice)

5. mice treated with X-rays + DEN

Treatment at an age of 7 days with a single dose of 0.50, 1 and 3 Gy followed 0.31, 0.62, 1.25 or 2.5 µg/g of DEN 7 days later (960 mice)

6. mice treated with DEN + neutrons

Treatment at an age of 14 days with 0.31, 0.62, 1.25 and 2.5 µg/g of DEN followed by a single dose of 0.12, 0.25 and 0.5 Gy of 3.1 MeV neutron 7 days later (460 mice)

7. mice treated with neutrons + DEN

Treatment at an age of 7 days with a single dose of 0.12, 0.25 and 0.5 Gy of 3.1 MeV neutron followed by 0.31, 0.62, 1.25 and 2.5 µg/g of DEN 7 days later (960 mice)

Autopsies were performed and the livers were removed in toto, weighed and inspected for the presence of grossly visible discoloration and for the number and size of nodular lesions. Sections of liver, 2 to 4 mm thick, were made through the longest axis of each lobe, fixed in buffered formalin and embedded in paraffin. Three µm thick sections were cut 200 µm apart and stained with haematoxylin and eosin. Four types of focal and nodular lesions were distinguished and recorded: foci, hyperplastic nodules, hepatocellular adenomas and hepatocellular carcinomas.
The focal lesions in 3-dimensional space were evaluated quantitatively from 2-dimensional liver intersections utilizing a MOP-VIDEOPLAN (KONTROL) image analyser. This computer was corrected to a transluscent digitizing table used either with a light cursor and a "Camera lucida" equiped Zeiss IM-35 microscope for direct measurement at microscopic resolution or as a screen for the projection of the whole section and macroscopic measurements. Calibration of the operating system was done using a calibration Zeiss Test-lines.

2. Results

Mice treated with X-rays

Choice of radiation doses: The choice of the X-ray doses to be used for the experiments on combined effects was based on a previous study on whole body irradiated adult C57BL male mice. The LD50 at 30 days was determined for mice exposed at 7 or 14 days of age and was respectively 5.87 and 5.78 Gy.

The causes of death in our normal control C57BL male mice are in the following decreasing order of frequency: 66% of non stochastic lung diseases and kidney diseases (glomerulosclerosis); 18% of the controls died from various types of leukaemias (e.g. thymoma 1.87%); 16% from carcinoma and sarcoma among which liver adenoma + adenocarcinoma (11%) were the most frequent.

Among the X-irradiated mice surviving longer than 30 days after increasing doses of X-rays, thymic lymphoma was most often (85.3%) responsible for death within 197 days. The optimal doses required to induce thymic lymphoma in the mice irradiated at 7 and 14 days of age were 6.5 and 5 Gy respectively. Thymic lymphoma was however rare in mice exposed to the doses of 0.5, 1 and 3 Gy used in the present experiment.

Diethylnitrosamine hepatocarcinogenesis in the infant mouse:

It was important to know to what extent our substrain of C57BL mice was susceptible to the induction of liver hepatomas by diethylnitrosamine. Since some strains and substrains are particularly prone to development of hepatomas, others are less susceptible. Genetic studies showed that the lethal genes Ay increase susceptibility to the extent that all male AyAF hybrid mice surviving for 16 months develop hepatomas. The susceptibility of a given species or strain of mice also varies with the particular carcinogen.

The results show that the C57BL/Cnb strain used is susceptible to the induction of liver cancer by DEN (Fig.1). The mice treated with the highest doses of DEN suffered from the most invasive lesions. The average number of liver lesions increased with doses of DEN. The latency period for the appearance of foci depends essentially on the dose of DEN. With the highest doses of DEN (2.5 μg/g) at 10 weeks after the beginning of the treatment each treated mouse presents lesions, their number increasing with time. For the lowest doses of DEN (0.3 μg/g) the latency period for the appearance of liver lesions is longer and waiting a period of 50 or 60 weeks, some mice among the 10 treated mice do not present any lesions.

Effects of X-rays combined with DEN on lesions induction in mouse liver

Combined treatment of DEN + X-rays:

Combined treatment of 0.31, 0.62, 1.25 or 2.5 μg/g given 7 days before increasing doses of X-rays showed that the development of liver lesions depends essentially on the dose of DEN and not on the dose of X-rays (Fig. 1 and 2). When the results obtained for a same dose of DEN and increasing doses of X-rays are compared, the results are not statistically different.

TOTAL AREA (DEN + Xray)

o—o RX= 0 Gy   ▲—▲ RX= 0.5 Gy   □— □ RX= 1 Gy   ▼· · ▼ RX= 3 Gy

a: DEN = 0.31 ug   b: DEN = 0.62 ug   c: DEN = 1.25 ug   d: DEN = 2.5 ug

Area (mm2)

Time after treatment (weeks)

Fig. 1. Development of liver lesions (foci, nodules, adenomas and carcinomas) in C57BL mice expressed in mm^2 of damaged liver tissue following a combined treatment of a given dose of DEN given 7 days before increasing doses of X-rays.

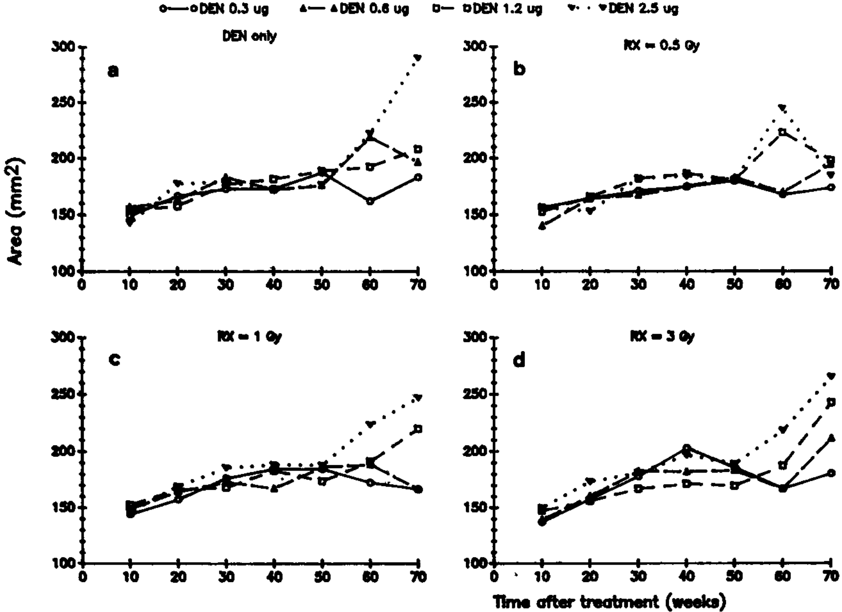

Fig. 2. Development of liver lesions (foci, nodules, adenomas and carcinomas) in C57BL mice expressed in mm^2 of damaged liver tissue following a combined treatment of increasing doses of DEN given 7 days before a given dose of X-rays.

Combined treatment of X-rays + DEN:

Combined treatment of X-rays given 7 days before increasing doses of DEN showed that the development of liver lesions behaved in the same way as the comparable group of mice receiving DEN before X-rays exposure (Fig. 3 and 4)

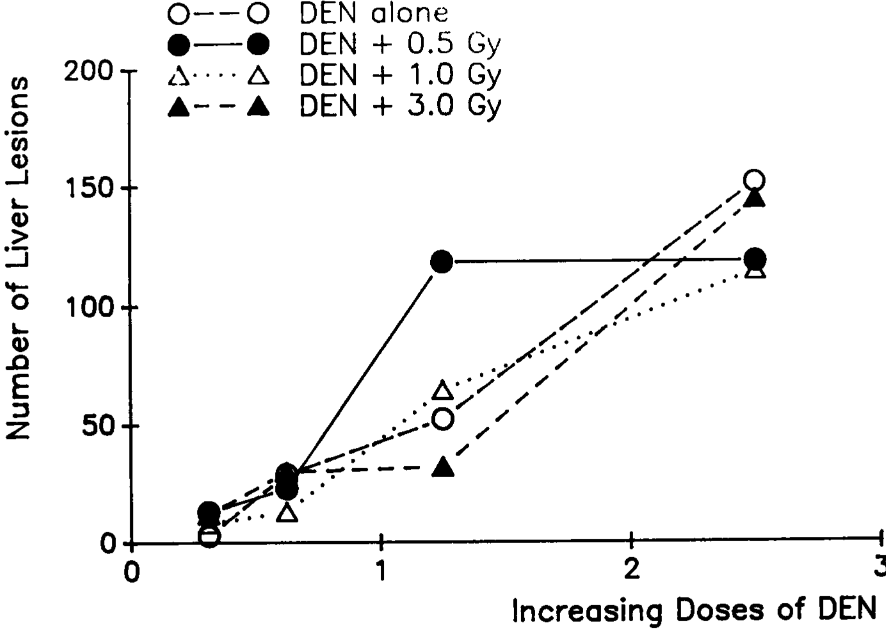

Fig. 3. Microscopic liver lesions (foci, nodules, adenomas and carcinomas) following a combined treatment of a given dose of DEN 7 days before increasing doses of X-rays.

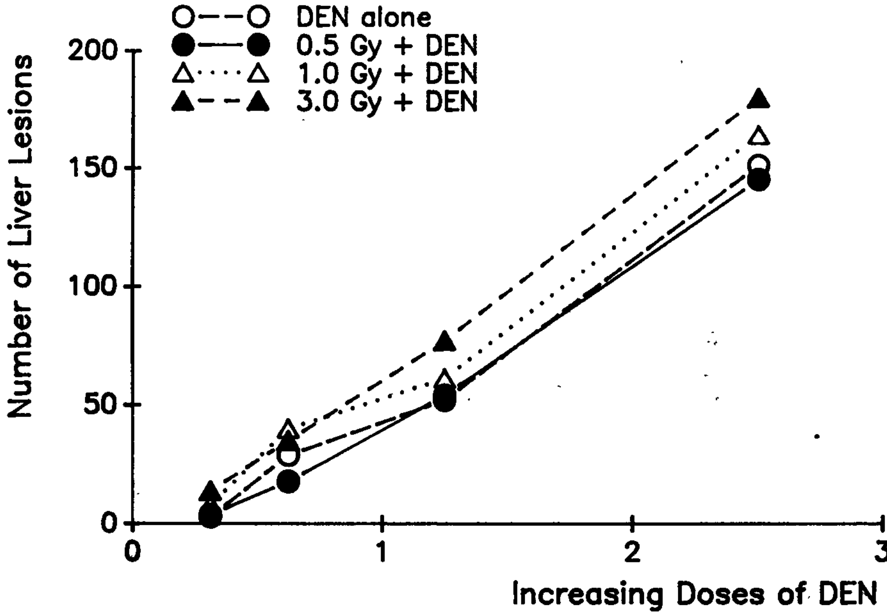

Fig. 4. Microscopic liver lesions (foci, nodules, adenomas and carcinomas) following a combined treatment of increasing doses of X-rays 7 days before increasing doses of DEN.

Mice treated with DEN + neutrons or with neutrons + DEN:

Unfortunately due to repair work at the Van Der Graef of the BCMN during the first part of the year 1988, the neutron irradiation had to be postponed until the end of the year 1988 and the first part of the year 1989. The preliminary results obtained show that neutron irradiation given 7 days before or after the administration of DEN does not have any additive or synergistic effects compared with the data obtained with DEN or neutron irradiated alone.

3. Discussion

There is a great scarcity of systematic data in which an analysis of combined effects can be based in spite of the large number of reports, where combined actions were tested and interactions claimed. There are at least three types of combined actions:
- additivity: (when the end-effect of the combined action equals the sum of the effects of the two agents acting independentely).
- synergism: (when the effects of the combined action exceed the sum of the effects produced separately by the agents).
- antagonism: (when the combined action results in an effect which is less than expected from the sum of the actions of the interacting agents).

In spite of the advantage of the liver system it has not been used very much for the study of the possible interactions of irradiations and chemicals. A synergistic action of fission neutrons with N,N-2-7-fluorenylenebisacetamide (2,7-FAA) was reported for irradiation of liver and gastric carcinoma but not for the intestinal tumours. Localized X-irradiation in association with 2,7-FAA administered in the diet accelerated the induction of hepatomas and similar effects were reported when X-rays were given with O-aminoazotoluene and of radioactive ^{144}Cs and dimethylaminoazobenzene (DBA). The infant mouse liver is much more easily transformed by chemical carcinogens than the adult liver. This may be due to the particular characteristics of infant liver which differ in several respects from the adult.

In a preliminary experiment we demonstrated that X-rays administered several days days before a single injection of DEN was more effective in inducing microscopic liver nodules than when administered 7 days after DEN administration (Maisin et al, 1986). Similarily in rats treated neonataly it was demonstrated that combining the DEN and radiation treatment, total focus yield was significantely increased although the response declined with the increasing radiation dosage. The author suggested that quantitatively different types of genetic damage (carcinogen-induced point mutation) and radiation induced rearrangements may interact synergistically in the induction of phenotypically altered cells. Our results show that the incidence of liver lesions after combined treatment of increasing dose of DEN for X-rays depends essentially on the dose of DEN, that the combined treatment of DEN and X-rays is not more effective than DEN given alone and that the dose of X-rays administered 7 days before a single injection of DEN is not more effective than when administered 7 days after DEN. Preliminary data on the induction of liver tumours after combined treatment of increasing doses of DEN or neutrons show similar results.

4. Conclusions

The incidence of liver lesions after combined treatment with increasing doses of DEN and X-rays depends essentially on the dose of DEN. The combined treatment of DEN and X-rays or of X-rays + DEN is not more effective than DEN given alone. Preliminary results obtained with neutron irradiation given 7 days before or after the administration of DEN seem not to present any additive or synergetic effects compared with the data obtained with DEN or neutron irradiation alone.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

V. Publications:

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- Oncogene 2 (1988) 215-222.

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The proviral genome of radiation leukemia virus: restriction enzyme analysis of an infectious molecular clone and sites of integration in tumor cell DNA.
- Arch. Intern. Physiol. Biochim. 92 (1984) B32.

J.R. MAISIN, L. de SAINT-GEORGES, M. JANOWSKI, M. LAMBIET-COLLIER, G. MATTELIN, J. VAN KERKOM
Effects of X-rays alone or combined with diethylnitrosamine on cancer induction im mouse liver.
- Radiation Research Society, North American Hyperthermia Group (1990).

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-C-073-F

Commissariat à l'Energie
Atomique, CEA
Département de Protection Sanitaire
B.P. n° 6
F-92260 Fontenay-aux-Roses

Head(s) of research team(s) [name(s) and address(es)]:

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Telephone number: 1-654.85.85

Title of the research contract:

Contribution of flow cytofluorimetry for the assessment of over-exposure to ionizing radiation.

List of projects:

Contribution of flow cytofluorimetry for the assessment of over-exposure to ionizing radiation.

Title of the project no.:

BI6-C-073-F

Contribution of Flow cytofluorimetry for the Assessment of Overexposure to ionizing Radiation

Head(s) of project:

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Scientific staff:

Frelat G. Ph D, Guilly MN. PhD, Schmitz A. Ph D, Guedeney G. Ph D, Grunwald D. Ph D, Mallarbet JL. P D, Prudhomme J. Technician, Chaput B. Technician.

I. Objectives of the project:

The evaluation of ionizing radiation overexposure has been made, until now, by measuring chromosomal aberrations in the mitotic lymphocytes of irradiated individuals. more recently, a method taking into account the radio-induced membrane modifications of different sub-populations of blood cells has been described.
Our aim is to adapt these valuable methods, but heavy and tedious, for flow cytometry analysis with the expected following benefits: rapidity, analysis of large number of biological objects, multi-parametric analysis, immediarte statistical analysis, correlations between different parameters.
The purpose of the project is to evaluate the potenciality of flow cytometry towards three main goals: heterogenous chromosomal aberration detection on flow karyotypes, use of anti-centromeric antibodies for detection of a- and dicentric chromosomes, and measurement of membrane modifications by fluorescent probes (lectins, monoclonal antibodies).

II. Objectives for the reporting period:

Work for the reporting period concentrated mainly on the labeling of chromosomal kinetochores. Different techniques were used in order to make it homogeneously staining chromosomes and to reduce background due to non specific staining by fluorescent antibodies.
Staining and detection of reticulocytes was performed using Thiazole Orange.

III PROGRESS ACHIEVED

The proposal involved three aspects:

1-Direct estimate of dose related damages to the irradiated chromosomes after exposure of lymphocytes in vitro.

2-Analysis of chromosomal aberrations by means of monoclonal antibodies.

3-Studies of radiation induced changes in the cell membranes.

1- DIRECT DETECTION OF CHROMOSOMES CHANGES USING FLOW CYTOMETRY.

The first two years have been devoted to technical investigations mainly dealing with improving cell culture and mitotic preparations. Computerized analysis of monoparametric signals related to DNA content (ethydium bromide) of each chromosome group has been proposed for three species: man, non human primates and pigs. High resolution biparametric scans of chromosomes have been tested by simultaneous recording of DNA content and relative concentration of AT/GC bases using Hoechst and Chromomycine A3. This last improvement allowed to separate 16-17 different peaks instead of 12 in the previous experiments for *Macaca fascicularis*, with the ATC 3000 flow cytometer.

The smallest chromosome of Macaca fascicularis was well separated from the background of cellular debris in a monovariate flow karyotype. It was thus possible to define a value of the background (BF) in this area normally free of chromosomes in relation to the number of analysed chromosomes. In 4 irradiation experiments on blood of 2 individuals, each experiment as well as the mean of the experiments showed a dose-effect relationship. However, if one wants to determine the value of the received dose from an isolated BF value through the mean standard curve, one can easily see some discrepancy in the results. This variability has certainly several causes: variability among the individuals and variability of the history of each individual (opposed to the stability of an in vitro cell culture), but certainly also the extreme dependance on the way chromosome suspensions are prepared.

These results, from blood isolated from two different individuals, are not very optimistic about the possibility of obtaining biological dosimetry when using such a method, contrary to the conclusions of the literature. However this method remains of some value for the detection of clastogenic agents in in vitro genetic toxicology tests. This general approach to study DNA damage generated by ionizing radiation does not seem to be promessfull for human biological dosimetry and so, we decided to analyse chromosomal abnormalities by a molecular approach involving flow sorting.

A recurrent t(11;22) breakpoint was mapped by chromosome flow sorting and spot-blot hybridization.

The breakpoint of the recurrent t(11;22) translocation, one of the most frequent chromosome anomalies encountered in human population, always involves bands 11q23.2 and 22q11.2. The involvement of the Cl locus of the immunoglobulin l gene cluster on chromosome 22 has been suggested: however, in situ hybridization experiments have yielded conflicting results. In order to solve these dicrepancies by another aproach, we have used bivariate flow sorting to separate the chromosomes of interest and to map the specific breakpoints by direct spot-blot hybridization with the gene-specific radiolabelled DNA probes *Alu*, Vλ, *ets*. The results showed unambiguously that in the t(11;22) patient analysed a set of Cλ and Vλ genes was tranlocated to the der(11) chromosome. Since Vλ genes are situated proximally to Cλ genes, we demonstrated that, in the case studied here, the chromosome 22 breakpoint in now located within or even immediately close to the Cλ region.

The transmission of a chromosome translocation has been followed in an experimental pig family

Again, the involved methodology was spot-blot hybridization after flow sorting. The efficiency of this method prompted us to determine if it was applicable to porcine gene localisation. The availability of individuals carrying a t(3;7)(p1.3;12.1) translocation favored this approch to confirm the assignment of MHC to porcine chromosome 7.

Oncogene detection by enzymatic amplification on flow sorted chromosomes

Direct spot blot hybridization on flow sorted chromosomes has allowed gene mapping and characterization of chromosomal rearrangements. But this thechnique still requires a relatively high number of sorted chromosomes and several concentrations in order to get an unambiguous positive signal especially when probing a single copy gene. The Polymerase Chain Reaction (PCR) was used to amplify directly on a few flow sorted chromosomes a specific sequence which can be then reliably identified. The results show that PCR reduces dramaticaly the requirement of flow sorted material for the detection of unique DNA sequence on specific chromosomes. So the combination of PCR and flow cytometry should enable the rapid and precise characterization of chromosomal rearrangements and oppens potential diagnostic applications.

2- ANALYSIS OF CHROMOSOMAL ABERRATIONS BY MEANS OF MONOCLONAL ANTIBODIES.

The feasibility of Kinetochore identification by imunostaining with autoantibodies secreted by scleroderma patients with CREST syndrome was evaluated during the first year of the contract. During the 2nd year, we demonstrated that a slight modification of labelling technique made it possible to stain chromosomes in suspension by indirect immunofluorescence with anti-centromere sera. However, we observed that a non specific fluorescence was revealed by the FITC-conjugated antibody and made the quantitative development of the method difficult.

Two human lymphoblastoid cell lines ICB 100 and 101 were used. Purified IgG and sera from patients with the CREST syndrome of scleroderma were the source of antikinetochore antibodies. The second FITC-labelled antibodies were anti-human IgG. All labelling was made on chromosomal suspensions. The flow cytometric analyses were carried out with dual-laser configuration for the detection of Hoechst-bound DNA fluorescence and the FITC-bound centromere fluorescence. Controls were only incubated with FITC labelled anti-human IgG.

Thanks to the possibility of analysing FITC fluorescence of the chromosome only, it was possible to detect the binding of antikinetochore antibodies to the chromosomes. Although we found no clear fluorescence intensity increase for a dicentric chromosome population obtained after cell culture irradiation, the existence of a tail on the FITC histogram precluded the detection of these dicentrics by flow analysis. Control experiments showed that the contaminent fluorescence seemed to be centromere non specific but chromosome specific. The presence of some chromosomes with higher level of FITC fluorescence may be due either to antibodies recognizing antigens other than the kinetochore or to kinetochores with different sizes varying with the chromosome lengths. The analysis by immunoblotting of the antigens recognized by the sera seemed to indicate the existence of some minor IgG able to recognize determinants other than the kinetochores..

Futher development were made during 1988 and 1989. Thanks to the courtesy of Dr. Earnshaw (Johns Hopkins University, Baltimore, Maryland, USA), 2 mouse monoclonal antibodies directed against CENP-B (one of the proteins of the kinetochore) were used for the centromere labelling of chromosomal suspensions from the two human lymphoblastoid cell lines ICB 100 and 101 previously employed (see "Euratom Progress Report 1987", p. 705-710).

These antibodies recognized different epitopes of a fusion protein obtained through the expression of a cDNA containing part of the gene CENP-B and part of the bacterial β–galactosidase gene (Earnshaw W.C. et al., J Cell Biol, 1987, 104: 817-829). The second fluorescent FITC-labelled antibodies were sheep anti-mouse IgG. Indirect immunofluorescence was also performed on HeLa cells grown on coverslips as described for the characterization of new nucleus specific autoantibodies (Guilly M.N. et al., Eur J Cell Biol, 1987, 43: 266-272). Various dilutions of these mouse monoclonal antibodies were tested on HeLa cells by indirect immunofluorescence microscopic analysis. A positive staining of kinetochores was only observed at low dilutions (1:10) contrary to human antiserum which still gave positive results at high dilution (1:10 000). Presently, none of these mouse monoclonal antibodies gave centromeric labelling on chromosomes in suspension with the method already used with human sera from patients with the CREST syndrome of scleroderma.

The negative results obtained on chromosomal suspension with these mouse monoclonal antibodies anti-CENP-B are understandable when comparing the difference in affinity observed on HeLa cells between them and human serum. The apparent low affinity of these mouse monoclonal antibodies may originate from trivial reasons such as lyophylisation/rehydratation difficulties. But as for the same human sera affinity differencies were observed between in situ and in suspension labelling techniques, it is very likely that the epitopes recognized on the fusion protein or on the cells grown on coverslips are more or less either hidden or modified when the chromosomes are released from the cells into the flow analysis buffer.

These results raise questions about the best biological reagents suitable for specific, high affinity, labelling of chromosome centromeres in suspension. It was thus decided, before searching for human monoclonal antibodies, to come back to human anti-kinetochore sera with 3 objectives: improvement of chromosomal suspension labelling method with new human antisera; characterization of human kinetochore proteins by immunoblotting and immunoprecipitation; purification of these proteins in order to produce new monoclonal antibodies and/or polyclonal sera.

Through collaboration with Dr F. Danon (Laboratoire d'Immunochimie et d'Immunopathologie, Hopital St Louis, Paris), 59 human sera from patients with the CREST syndrome of scleroderma were available. Indirect immunofluorescence was performed, as described in the preceeding section, on HeLa cells grown on coverslips, except that the second fluorescent FITC-labelled antibodies were sheep anti-human IgG. For biochemical characterization of the sera, immunoblotting and immunoprecipitation of nuclear proteins from KE37 cells were performed as reported for lamins (Guilly et al., 1987, see above).

All of the 59 sera were tested at various dilutions on HeLa cells. Though all were characterized as antikinetochore-specific by immunofluorescence analysis, the FITC background differed from one serum to another. Biochemical characterization of 17 sera was undertaken by performing immunoblotting and immunoprecipitation of the recognized nuclear antigens from a human T lymphoblastoid cell line (KE37). On immunoblots, most of the sera (11/17) detected the 80 kD nuclear protein (CENP-B). The antibodies eluted from this nitrocellulose-immobilized antigen labelled the kinetochores on cultured cells. However an important background level and a strong non-specific staining of the histones were often observed with this technique. Immunoprecipitation of biosynthetically labelled nuclear proteins was carried out to overcome the problem of non-specific binding. This method allowed the identification of 8 other proteins having the following molecular weights: 21, 51, 65, 70, 75, 110, 150 and 200 kD, which were also recognized by some of the 17 CREST patients' sera.

The variation of FITC background between sera reinforces the need to select the best one and to determine accurately the optimal concentrations for autoimmune sera and FITC-labelled second antibodies. The results from immunoblotting and immunoprecipitation experiments show that antigens other than the 80 kD protein may be recognized by the patients' sera. As the mercury arc lamp used in fluorescence microscopy has a poor exciting capacity for FITC, the human eye cannot distinguish very low fluorecence level which can be revealed by laser illumination. For example, the detection limit of the flow cytometer used (ATC 3000) is 700 true FITC molecules (Gaucher et al.,1988, this study). Therefore it is important to characterize biochemically the antigens recognized by the CREST sera before looking for monoclonal or polyclonal antibodies. In this respect the immunoprecipitation of biosynthetically labelled nuclear proteins seems to be a good technique. The obtention of pure antigens from chromosomal centromeric regions may solve the necessity of a choice between monoclonal antibodies (specific, low affinity) and polyclonal antibodies (less specific, high affinity). Another approach could be the use of a slit-scan flow cytometer which allows the recognition of the fluorescence profile of each chromosome. The possibilty of setting a fluorescence intensity threshold should permit the unambiguous detection of dicentric chromosomes.

During the last year of the contract, a cooperative research has been developed with Barbara Trask at Lawrence Livermore National Laboratory on the DBC Flow cytometer prototype in order to optimize the discrimination of these low intensity fluorescence signals. Sera were selected on the basis of the lowest background and a method for separating labeled chromosomes from unbound antibodies, debris and nuclei was developed: centrifugation on a Ficoll 400 discontinuous 12-25% gradient. Flow analysis of centromere labeled chromosomes stained for AT/GC bases content, showed that:

1- Flow karyotype resolution was maintained after staining procedure.

2-The centromeres specific-FITC fluorescence intensity, was estimated to be 8 times higher than the background fluorescence in the case of human, and even higher for hamster.

3-There is no proportionality between the FITC signal and the DNA content. Thus, the FITC signal can be considered as strongly centromere related.

These last results showed unambiguously that human anti-kinetochore antibodies should allow quantitative detection of centromeric material.

3- RADIATION INDUCED CHANGES IN CELL MEMBRANES

Samples taken from human donnors and from Sprague Dawley rats were used and prepared for binding with concanavalin A (ConA) labeled with Fluorescein isothyocyanate (FITC), after a slight fixation with paraformaldehyde. Fluorescence intensity was recorded after excitation at 488nm on a ATC 3000 flow cytometer. The detection was found feasible despite some variability, partially reduced in fixed cells, as shown by introduction of an internal control made of florescent beads. Fluorescence intensity plateaued for concentration of FITC between 100 and 150mg of ConA per million of cells.

Rats were exposed to a cobalt source in vivo to 0, 0.5, 1, 2 Gy at a dose rate of 0.25 Gy per minute. Resulting fluorescence was analysed 2 hours after exposure in the different blood cell subtypes.

Results were constantly negative in that sense that radio-induced changes were not discernable from inter-individual variability. Moreover, changes were unpredictable, receptor sites being increased or decreased in number for similar exposure conditions.

In conclusion, as observed by other authors, and notely by Dehos, Radiation Protection Programme progress report 1987, we can not recommend the use of recptors for ConA lectin as an indicator for over-exposure to ionizing radiation.

GENERAL REMARKS

Despite flow cytometer is a very powerful tool, there are still progress to be made before it can be used under operational conditions for sorting irradiated people.

It would be unexpected if a direct recording of the karyotype in flux could reveal, in individuals, a reliable dose dependant relationship due to clastogenic effects. Despite this limit, the feasibility of translocation detection was shown and gene amplification with PCR should become a powerfull tool to detect radiation induced genetic damages.

Membrane receptors to lectins have not been validated in flux up to now.

The most promissing method remains centromere identification by human antibodies which will probably allow to detect dicentric chromosomes. Production and purification of the reagent are still needed and validation of the process still calls for slit scan devices.

Some implementations are already known as feasible like mutation at the glycophorin locus for red blood cells, other may be of great interest like reticulocyte counting that we develop for simpler devices in the laboratory. Attention should also be paid to telomeric fraction of the chromosome. It is known as a region of DNA required for replication and stability of the chromosome; it may be involved in assymetrical exchanges as dicentrics and arguments (Chadwick and Leenhouts) have been given that telomere can rejoin a broken end of another chromosome. Preliminary results in our hands have shown that telomeres and centromeres are involved in large scale chromosome rearrangements as shown by a repetitive sequence of DNA specific for hamster telomere and centromere.

The centric regions of human chromosomes contain a heterogeneous mixture of highly repetitive DNA sequences families, one of which is present in the centromere of all human chromosomes (alpha satellite DNA). This sequence can be used as a probe for further development of the technique.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

USA: Laurence Livermore National laboratory, Biomedical Biology Division : Dr Barbara **Trask**
France: Institut Curie, Laboratoire de Génétique des Tumeurs: Dr Bernard **Dutrillaux,** Dr Gilles **Thomas**
Hôpital St Louis PariLaboratoire d'immunochimie et d'immunopathologie, Drs F **Danon** et JC **Brouet**
CEN Saclay, Département d'Electronique Industrielle et Nucléaire: Drs JC **Gaucher** et A. **Seigneur**

V. Publications:

Grunwald D., Geffrotin C., Chardon P., **Frelat G., Vaiman M.**
Swine chromosomes : flow sorting and spot blot hybridization
Cytometry, **7**, 582-588, 1986.

Delattre O, Grunwald M., Bernard A., **Grunwald D.,** Thomas G., **Frelat G.,** Aurias A.
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In: "Flow cytometry: Advanced Research and Clinical applications". A. Yen (ed.). CRC Press Inc. Boca Raton. USA. Vol. I Chap. 7, pp 131-142, 1989.

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Oncogene detection by enzymatic amplification on flow sorted chromosomes.
Nucleic Acid Research, **17**, pp 816, 1989.

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"Flow sorting of swine chromosomes: confirmation of MHC mapping on chromosome 7"
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"Caryotype en flux de tumeurs solides humaines: étude du sarcome d'Ewing"
(2éme Colloque de l'Association de Cytométrie en Flux: "Applications Cliniques de la Cytométrie en Flux". Rennes. 28-29 Novembre 1985)
Biology of the Cell, 55, 12a

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Biology of the Cell, 55, 8a

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"Swine chromosomes: Flow sorting and spot-blot hybridization"
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Cytometry, 8 (supl. 1), 13.

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(6ème Congrès Annuel de l'Association de Cytométrie en Flux: "La cytométrie en flux: analyse multiparamétrique". Lyon. 21-22 Septembre 1989.
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Biology of the Cell, 67, 28a.

RADIATION PROTECTION PROGRAMME

Progress Report

1989

Contractor: Contract no.: BI6-C-310-F

C.E.A.
I.P.S.N.
Département de Protection Sanitaire
B.P. N° 6
F- 92260 Fontenay-aux-Roses

Head(s) of research team(s) [name(s) and address(es)]:

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Title of the research contract:

Foetal dosimetry: measurement of the effects induced after in utero chronic irradiation as a function of dose rate and gestation age

List of projects:

1. Foetal dosimetry: measurement of the effects induced after in utero chronic irradiation as a function of dose rate and gestation age

Title of the project no.: B16-C-310-F

Fetal dosimetry : measurement of the effects induced after in utero chronic irradiation as a function of dose rate and gestation age.

Head(s) of project:

Dr H. Métivier
SPE/STCE/LTT, BP 12 91680 Bruyères le Chatel, France.

Scientific staff:

H. Coffigny, P. Fritsch and M. Beauvallet.

I. Objectives of the project:

The aim of this project is to estimate irradiation doses delivered to the embryo or the fetus after internal contamination by radionuclides. For that purpose, we determine a dose-effect relationship between the irradiation doses delivered by protracted external irradiation during the whole intra-uterine life or part of it, and biologicals effects measured on different radiosensitive target organs. Thus, irradiation doses delivered after internal contamination could be estimated from the measurement of the induced biological effects.

II. Objectives for the reporting period:

In order to estimate the irradiation doses delivered to the embryo and the fetus after internal contamination, the effects of protracted gamma whole body irradiation during the whole intra-uterine life of rodents or part of it were studied . For a given dose rate delivered during the whole intra-uterine life, different early or late effects induced by the external irradiations were analysed to determine the effects for which a clear dose-effect relationship could be observed as a function of the delivered dose. For these effects, we have studied the influence of dose rates and we have estimated the intra-uterine periods of their induction.

III. Progress achieved:

Methodology

Rats and mice were irradiated by 60Co gamma rays (>23 hours per day) during the whole intra-uterine life and two groups of mice were irradiated during the last 2/3 or last 1/3 of gestation. Dose rates were 3, 10, 25 and 37 cGy /day in rats and 20, 40 and 60 cGy/day in mice. Four groups of rat fetuses received a 1 Gy acute irradiation on day 15 p.c. at doses rates of 0.86, 4.75, 23.9 and 239 Gy/day.

Early effects were studied the last day of gestation and late effects at 3 months of age. Intra-uterine and post-partum mortality, body and brain weights were measured. Brain histology was analysed.

Results

Except for a dose rate at 60 cGy/day delivered during the entire gestation, the irradiations did not increase intra-uterine lethality as compared with controls. In mice irradiated at 40 cGy/day during the whole gestation or at 60 cGy/day during the last 2/3 of gestation, all pups died during the neonatal period whereas no lethality was observed for lower dose rates. In rats and mice, the fetal weight measured on the last day of gestation decreased as a linear function of the delivered dose or dose rate. Relative brain weight also showed a significant decrease for the largest delivered doses.

In adults, body weight of irradiated rats and mice was similar to controls. On the contrary, the relative brain weight decrease was larger in the adults than in the fetuses. Brain weights decreased as a linear function of doses or dose rate. The rest of the brain (mesencephalum and cerebellum) was poorly altered by the irradiations. In mice, the brain weight drop was similar when irradiation was delivered during the whole gestation or during the last 1/3 of gestation.

The brain weight decrease induced by 1 Gy acute irradiation varied as a function of dose rate for rates larger than 4.75 Gy/day. Gros malformations were only observed for these largest dose rates.

Discussion

Fetal body weight and adult brain weight are good parameters to estimate the dose delivered during the protracted irradiation of rats and mice during their intra-uterine lifes but are restricted to unlethal doses . In the range of dose rates up to 60 cGy/day these weight alterations are not dose rate dependant.

By contrast, after a1Gy acute irradiation delivered on day 15 p.c., a specific dose rate effect was observed in adults as concerned brain weight for dose rates larger than 4,75 Gy/day. Further studied are needed to characterize this dose rate effect on brain and to determine if this dose rate effect could be induced in other target organ .

IV. Objectives for the next reporting period:

The objectives for the next reporting period are:
-to complete the histologic studies on brain for a better characterization of the dose rate effect
-to study other target organs such as gonads .

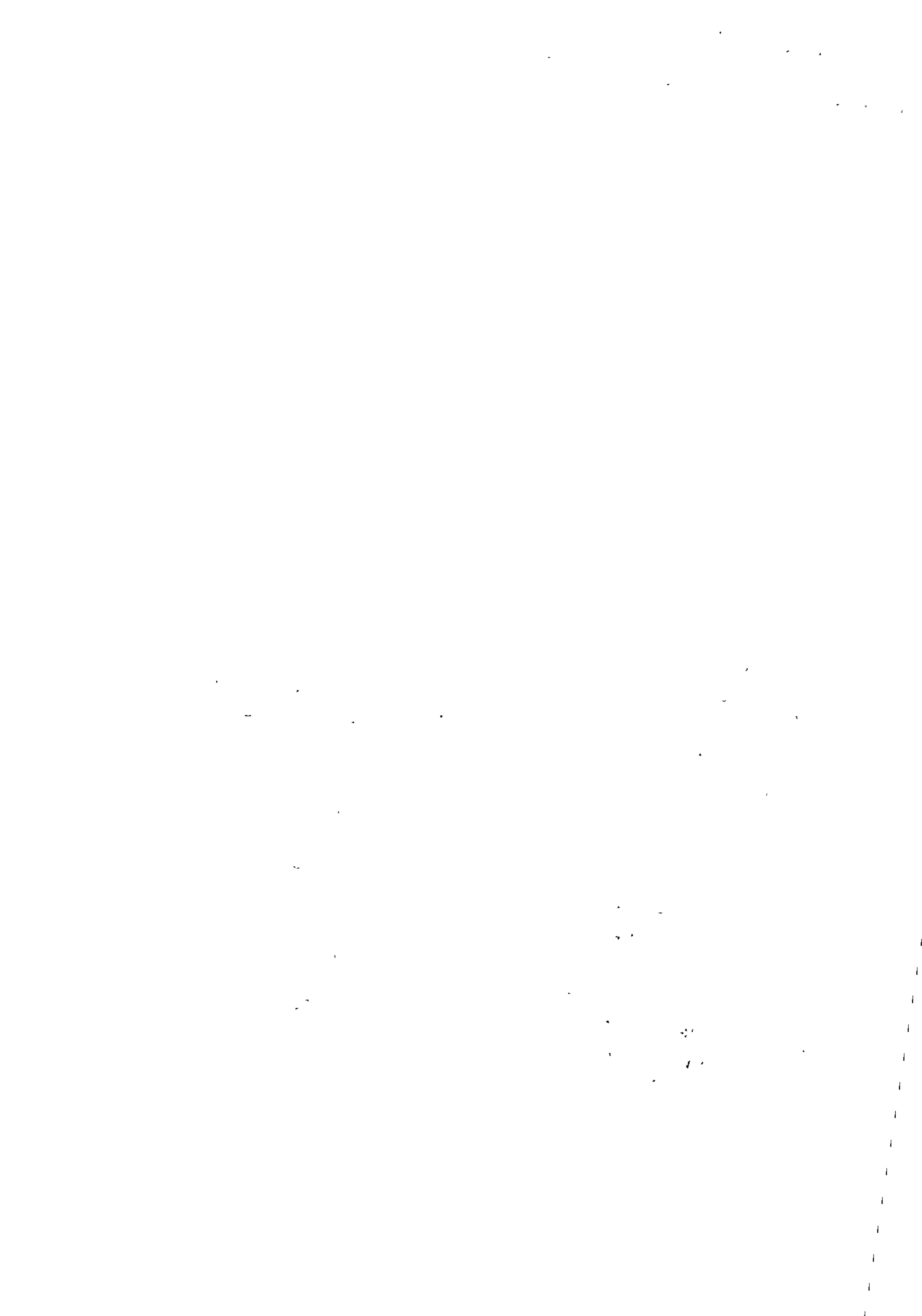
V. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

VI. Publications:

* Coffigny H., Fritsch P., Vernois Y., Beauvallet M., Court L., Métivier H. and Masse R.
Influence of dose rate on the biological effects of protracted gamma irradiation during the whole gestation of rats. EULEP Newsletter, 1988, 50, 28-29.

* Fritsch P., Coffigny H., Vernois Y., Beauvallet M., Court L., Métivier H. and Masse R.
Early and late effects of protracted gamma irradiation during intra-uterine life. EULEP Newletter, 1989, 52, 26-27.

* Coffigny H., Fritsch P., Beauvallet M., Court I., Métivier H. and Masse R.
Dose-rate effect of gamma irradiation during the intra-uterine life on brain development. 22nd Annual Meeting of the European Society for Radiation Biology. Brussels, Belgium, September 11-16, 1989.



RADIATION PROTECTION PROGRAMME
Final Report

Contractor:

Contract no.: BI6-C-074-UK

United Kingdom Atomic Energy
Authority
11 Charles II Street
GB-London SW1Y 4QP

Head(s) of research team(s) [name(s) and address(es)]:

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Env. & Med. Sciences Division
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Telephone number: 0235-24141 (Ext. 4622)

Title of the research contract:

Macrophage involvement in actinide-induced lung disease.

List of projects:

1. Macrophage involvement in actinide-induced lung disease.

Title of the project no.:

Macrophage involvement in actinide-induced lung disease

Head(s) of project:

Dr A Morgan

Scientific staff:

Dr ND Priest, Dr A Taya, Mr RJ Talbot, Mr JP Kellington.

I. Objectives of the project:

To study the effects of alpha-emitting transuranic nuclides on the alveolar macrophage and the use of this cell as a marker for investigating early radiation effects on the lung. To extend this work to lung epithelial cells.

II. Objectives for the reporting period:

1) To study the effects of inhaled α emitters on macrophage kinetics.

2) To assess functional changes in the alveolar macrophage induced by inhaled α-emitters.

3) To study the induction of nuclear aberrations in alveolar macrophages, particularly at low radiation doses.

4) To initiate work on the effect of α emitters on alveolar Type II pneumocytes and Clara cells.

III. Progress achieved:

1. Effects of inhaled α emitters on alveolar macrophage kinetics

1.1 Methodology

The use of ^{3}H-labelled thymidine and autoradiography to detect AM in S-phase is not possible following the inhalation of α emitters because the silver grains produced in the photographic emulsion by ^{3}H are obscured by α tracks. We have therefore developed an alternative technique which involves the incorporation of bromodeoxyuridine (BrdU), an analogue of thymidine, into the DNA of dividing AM. The BrdU-substituted DNA is visualised by an immunocytochemical technique using a fluorescent labelled monoclonal antibody against BrdU.

1.2 Results

Using the BrdU technique, it was established that in normal CBA mice, approximately 1.7% of AM recovered by broncho-alveolar lavage are undergoing DNA synthesis. In addition, it has been shown that less than 1 in 1000 AM are in mitosis. Additional studies in normal CBA mice showed that there was no significant change in the DNA labelling of AM over a period of 24 hours, indicating that there is no diurnal variation.

To investigate the effect of α emitters on DNA synthesis in AM, mice were exposed to $^{239}PuO_2$ to give initial alveolar deposits (IADs) of 60 and 400 Bq. The proportion of AM in S-phase at various times after exposure are shown in Fig. 1.1.

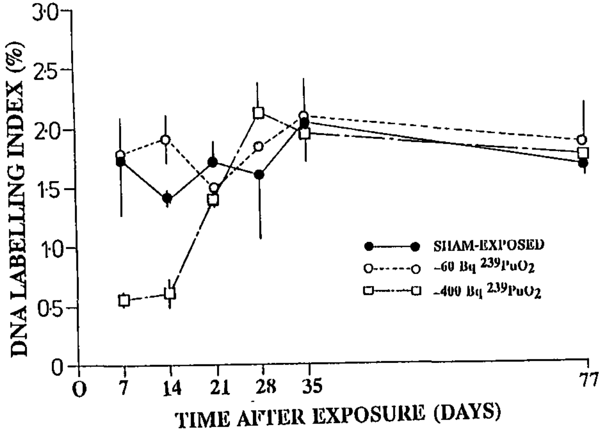

Fig. 1.1 Proportion of alveolar macrophages in S-phase following exposure to $^{239}PuO_2$.

1.3 Discussion

The preliminary work with control CBA mice showed that the alveolar macrophage is not a terminally-differentiated cell incapable of further division and that, under normal conditions, approximately 1.7% of AM recovered by lavage are undergoing DNA synthesis. This value compares well with the results of other studies using ^{3}H-labelled thymidine.

Studies of AM kinetics following exposure to $^{239}PuO_2$ (see Fig. 1.1) showed that the number of AM in S-phase is depressed for a period of about 2 weeks following exposure to $^{239}PuO_2$ to give an IAD of 400 Bq. This inhibition of the normal cell cycle accounts, at least in part, for the reduction in the number of AM which occurs over this period. The subsequent increase in the number of AM coincides with the increase of cells in S-phase. The results of this study are consistent with the theory of an intrapulmonary pool of proliferating AM, or AM precursors.

2. Functional changes in pulmonary alveolar macrophages following exposure to $^{239}PuO_2$

2.1 Methodology

The clearance of insoluble particles from lung is mediated by the alveolar macrophage (AM). Previous studies have shown that the mobility of AM which had taken up $^{239}PuO_2$ in vitro was impaired. The objective of the present study was to investigate the effect of inhaled $^{239}PuO_2$ on the phagocytic competence of AM using fluorescent polystyrene microspheres (FP). Initial studies were carried out to determine the relationship between the conditions of exposure (concentration of FP and exposure time) and the distribution of FP in AM. Mice were exposed to a constant concentration of FP and groups were withdrawn from the exposure chamber after 5, 20, 75 and 165 min. Animals from each group were killed at 1 and 8 d after exposure and AM recovered by broncho-alveolar lavage *in situ*. Using cytocentrifuge slides, measurements were made of the labelling index (LI - proportion of AM which contain FP) and the mean number of FP per labelled AM (LAM). From this information, the mean number of FP per AM was calculated.

In the study with $^{239}PuO_2$, mice were exposed by inhalation to give an IAD of 250 Bq. At subsequent time points (5, 19, 33 and 96 d post-exposure) groups of mice were exposed to FP by inhalation and killed

2 d later when the lungs were lavaged. LIs for AM recovered from control and ^{239}Pu-exposed mice were compared.

2.2 Results

The LIs for AM recovered from mice exposed to FP for varying periods are shown in Fig. 2.1 and the numbers of FP per AM in Fig. 2.2.

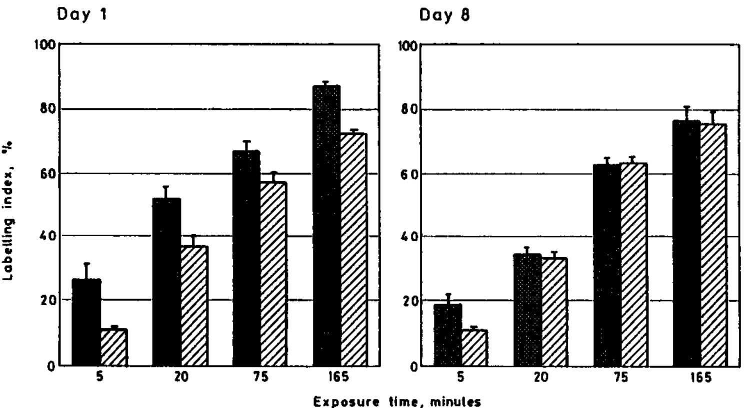

Fig. 2.1 Labelling indices of AM recovered by lavage at 1 ■ and 8 ▨ d after exposure.

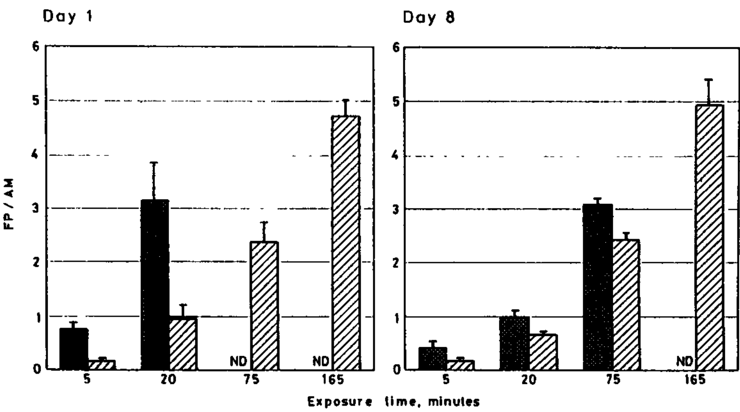

Fig. 2.2 Mean number of FP per AM at 1 ■ d and 8 ▨ d post-exposure. (Some of the AM recovered in washes 1 and 2 contained too many FP (>50) to assess accurately and in these groups measurements were restricted to washes 3-10).

The LIs of AM recovered from mice which had been exposed to $^{239}PuO_2$, and killed following exposure to FP, are shown in Table 2.1.

Time (d)	Control	$^{239}PuO_2$
7	49.1±10.1	55.4±4.1
22	49.9± 2.4	51.3±8.7
35	46.3±26.6	50.4±9.6
98	47.6± 4.7	46.7±0.8

Table 2.1 Percent of recovered macrophages containing fluorescent particles following exposure to $^{239}PuO_2$. Mean ± SEM.

Mice from this group, and from a sham-exposed control group, were killed at 7, 21, 35 and 98 days following exposure to $^{239}PuO_2$ to give an IAD of 250 Bq. Two days before sacrifice, mice were exposed to airborne FP. The lungs were lavaged with physiological saline to recover AM and the recovered cells were analysed by flow cytometry (FACS). In addition, cytospins of AM were prepared so that the results obtained by FACS could be compared with those obtained by manual scoring of cells using a conventional microscope equipped with epifluorescence.

2.3 Discussion

Fig. 2.1 shows that, at 1 d post-exposure, LIs were significantly greater for washes 1 and 2 than for washes 3-10, but at 8 d these differences had largely disappeared. Fig. 2.2 shows that mean numbers of FP/LAM at 1 d were much greater in cells recovered in washes 1 and 2 than in washes 3-10. The differences between 1 and 8 d are due to the presence of a relatively few heavily-loaded LAM which were recovered in the initial washes. The preferential recovery of these heavily-loaded cells, and the fact that most had been removed from the lungs by 8 d, suggest that they are situated either in the smaller conducting airways or in alveoli adjacent to the terminal bronchioles and have picked up particles deposited preferentially at these sites. The mean numbers of FP per AM were linearly-related to exposure time which indicates that there

was no saturation of the phagocytic capacity of AM. We conclude from this study that, in terms of particle distribution, AM recovered in washes 3-10 are more representative of the general AM pool than cells recovered in washes 1 and 2. The relationship between LI and FP/AM for washes 3-10 at 8 d is shown in Fig. 2.3 from which it can be deduced that, if the AM pool contains immature non-phagocytic cells, the proportion of such cells is small.

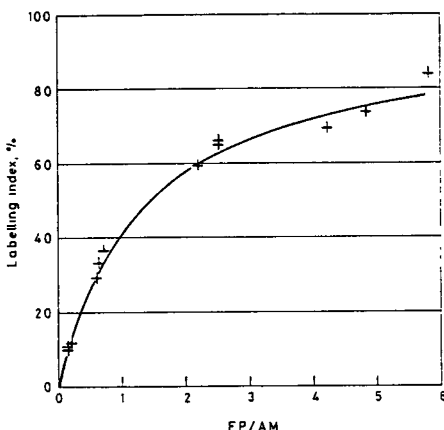

Figure 2.3 Relationship between LI and number of FP/AM in mice exposed for 5-165 min. Results for combined washes 3-10 at 8 d after exposure.

In mice exposed to $^{239}PuO_2$ followed by FP, the differences in labelling index between experimental and control mice were not significant at any time point (Table 2.1). Indeed, the LI in experimental mice at 7 d appeared to be greater than in controls, possibly because of the reduction in AM numbers at this time. Examination of cytocentrifuge preparations showed that, in ^{239}Pu-exposed mice, more macrophages contained >30 FP than in controls. Subsequent autoradiography showed that cells which contained many FP also contained a relatively large ^{239}Pu activity. Thus it appears either that AM containing $^{239}PuO_2$ have an enhanced phagocytic activity, or that both $^{239}PuO_2$ and FP are deposited preferentially at favoured sites within the lung and are taken up by AM at these sites.

3. Induction of nuclear aberrations at low doses

3.1 Methodology

Previous studies (Morgan et al. 1989) showed that the induction of nuclear aberrations in alveolar macrophages is a sensitive indication of radiation damage. In that study, IADs of 21-1660 Bq were used and the incidence of nuclear aberrations scored on cytocentrifuge preparations. Recently, this investigation was extended using IADs down to <1 Bq.

3.2 Results

All the measurements were made on AM recovered by lavage at 35 days after exposure to $^{239}PuO_2$. Estimates of the total yield of AM with micronuclei (MiAM) are shown in Fig. 3.1.

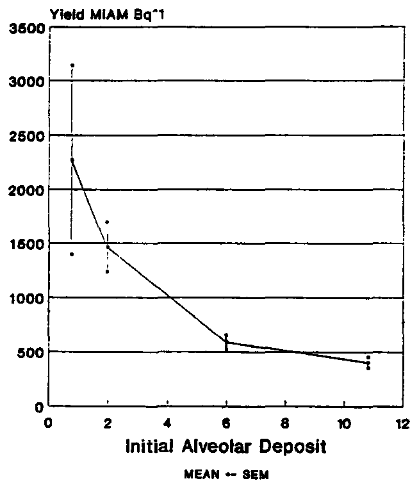

Fig. 3.1 Total numbers of MiAM per Bq IAD of $^{239}PuO_2$ at 35 d post-exposure

3.3 Discussion

The incidence of AM with micronuclei recovered from sham-exposed control mice was less than 0.1%. Even at the lowest IAD (0.76 Bq) both the incidence and estimated total number of MiAM were greater than in control mice and demonstrates the sensitivity of this test as an index of cytogenetic damage to AM.

Fig. 3.1 shows how the yield of MiAM per unit dose increases rapidly wih decreasing dose. In a previous study the yield of MiAM per unit dose at the lowest IAD (21 Bq) was approximately 370. This value compares well to a yield of 480 MiAM per Bq at the highest IAD (10.8 Bq) in the recent study.

Micronuclei are not expressed until a cell has divided at least once. The results of this study probably indicate the increased death of AM with cytogenetic damage, and an inhibition of AM division after exposure to higher doses of ^{239}Pu.

4. Effects of alpha emitters on lung epithelial cells

During the period under review, the focus of work has shifted from the alveolar macrophage to lung epithelial cells (Type II pneumocytes and Clara cells) which, unlike the highly differentiated alveolar macrophage, are thought to be tumour progenitor cells. The main objective has been to develop techniques for studying changes in epithelial cells following the inhalation of insoluble alpha emitters and radon/radon daughters. The techniques which have been developed include the following:-

- Gross morphology using plastic lung sections including methods for quantifying subtle morphological changes at the light microscope level.
- Ultrastructural changes using TEM.
- Biochemical determinations including enzymes, protein and cytokines (interleukin) levels in lavage fluid.
- Expression of oncogenes, cytokeratins and lectins during the development of lung tumours.

Preliminary results of a short-term study, which involved exposure of mice to $^{239}PuO_2$ to give an initial deposit of 500 Bq, showed that there was an increase in the proliferation of epithelial cells, particularly Type II cells, between 4 and 8 weeks after exposure. Hyperplastic changes in Type II cells were also observed. These studies will be extended to lower levels of alpha emitters in long-term studies. The results will contribute to better understanding of the mechanisms by which alpha emitters cause pre-neoplastic damage to the lungs.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

None.

Publications

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-C-068-D

Gesellschaft für Strahlen-
und Umweltforschung mbH
Ingolstädter Landstr. 1
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Head(s) of research team(s) [name(s) and address(es)]:

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Institut für Pathologie
GSF
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Telephone number: 89-3187.2538

Title of the research contract:

Morphological and immunclogical characterization of cells from typical focal CNS lesions in the rat following prenatal X-irradiation and their relationships to ethylnitrosourea neurocarcinogenesis.

List of projects:

1. Morphological and immunological characterization of cells from typical focal CNS lesions in the rat following prenatal X-irradiation and their relationships to ethylnitrosourea neurocarcinogenesis.

Title of the project no.: 1

Morphological and immunological characterization of cells from typical focal CNS lesions in the rat following prenatal X-irradiation and their relationships to ethylnitrosourea neurocarcinogenesis.

Head(s) of project:

Dr. W. Schmahl

Scientific staff:

Dr. J. Plendl

I. Objectives of the project:

This project is concerned with the post-irradiational membrane properties of the neuroglioblasts in the rat fetus. These cells represent the stem cell population for the development of the central nervous system. An ordered sequence of cell division, neuron migration and settlement depends largely on functional membrane constitution, thus influencing intercellular contacts, cell communication and pattern recognition. Alterations of these membrane properties by prenatal irradiation are suggested to modify the response of single cells or of the tissue as a whole to a potential carcinogenic agent, like ethylnitrosourea (ENU).

II. Objectives for the reporting period:

One important factor for inducing late effects following either X-irradiation of the fetus or in response to ethylnitrosourea (ENU) treatment is the magnitude of DNA repair. In the present experiments we analyzed the DNA repair capability of the fetal brain and liver simultaneously with long-term observations of rats which were treated in utero either by X-irradiation, ENU, or both in combination. This should reveal any relationship between the influence on O^6-alkylguanine-DNA alkyltransferase activity, which is the specific acceptor protein in DNA repair process, and the incidence of tumours in the offspring.

III. Progress achieved:

1. METHODOLOGY

1.1. Prenatal tissue sampling

14 pregnant rats were divided into 6 groups and treatment schedules as shown in Table 1. The dams were killed under CO_2-inhalation on day 17 of gestation between 10 a.m. and 12 a.m. The fetal brains and livers were rapidly removed, as well as the maternal brains and a part of the liver. The pooled organs were homogenized at 4°C, sonicated and centrifuged at 28000 g for 30 min. for tissue extraction. The transfer of ^3H-labelled methyl groups from the O^6-position of guanine in DNA to the acceptor protein was determined from the appearance of tritium in an insoluble protein fraction (procedure as described by Myrnes et al., *Carcinogenesis 5, 1061-1064, 1984*).

1.2. Postnatal long-term observation

18 pregnant rats were treated as described in Table 1. The offspring were observed until 26 months of age. In addition to macroscopical observation all brains were observed histologically by serial sections of the forebrains.

2. RESULTS

2.1. In the brain of the rat fetuses the content of the transferred O^6-methyl-guanine of the controls was 6.4 ± 2.3 fmol/250µg extract protein. This represents 100.0 ± 35.9 % alkyltransferase (AT) activity as shown in Table 2. The AT activity dropped to 60.9 ± 23.0 % of untreated controls after ENU treatment (group 4). A dose of 1 Gy induced a slight increase in AT activity compared to controls (131.3 ± 23.8 %, group 2) and a dose of 2 Gy induced a clear induction of AT (201.6 ± 21.7 %, group 3). The combination of X-irradiation pretreatment and ENU injection reduced this increase to 103.1 ± 34.8 % at a dose of 1 Gy and to 157.8 ± 25.7 % at a dose of 2 Gy. By contrast, the AT in the liver of the rat fetuses did not react in such a clear manner. Only a single dose of 2 Gy resulted in an increase to 142.3 ± 13.4 % of controls.

2.2. There were no brain tumours either in controls or in X-irradiated offspring. ENU (50 mg/kg) induced gliomas in 15 of 34 offspring (= 44.1 %) with a mean multiplicity of 2.13 per affected animal. Pre-irradiation with 1.0 Gy dropped the glioma frequency to 26.8 % (multiplicity 2.18). In those offspring which were X-irradiated with 2.0 Gy before ENU application, we

Table 1: Pattern of treatment and numbers of the treated rats

Group No. Treatment (gestation day 16)	1 Controls	2 X-irrad. 1.0 Gy	3 X-irrad. 2.0 Gy	4 ENU (50 mg/kg)	5 X-irrad. plus ENU 1.0 Gy	6 X-irrad. plus ENU 2.0 Gy
I Prenatal tissue sampling						
No. of dams	2	3	3	2	2	2
No. of fetuses	23	37	35	24	25	22
II Postnatal tissue sampling						
No. of dams	2	3	3	3	4	3
No. of fetuses	22	32	25	34	41	24

Table 2: AT activity in brain and liver after application of ENU alone or in combination with X-irradiation on gestation day 16 of the rat

Group No.	AT activity (%) in the fetal brain	in the dam's brain	in the fetal liver	in the dam's liver
1 (Controls)	100.0 ± 35.9	100.0 ± 3.6	100.0 ± 1.8	100.0 ± 11.6
2 X-irrad. 1.0 Gy	131.3 ± 23.8 [b]	146.4 ± 19.5 [c]	97.7 ± 16.7	127.7 ± 16.7
3 X-irrad. 2.0 Gy	201.6 ± 21.7 [c]	171.4 ± 16.7 [c]	142.3 ± 13.4	157.4 ± 11.5
4 ENU	60.9 ± 23.0	69.6 ± 7.7	95.5 ± 18.5	112.9 ± 4.0
5 X-irrad. 1.0 Gy plus ENU	103.1 ± 34.8 [a]	116.1 ± 10.7 [c]	113.6 ± 12.4	149.0 ± 3.0
6 X-irrad. 2.0 Gy plus ENU	157.8 ± 25.7 [c]	142.9 ± 7.5 [c]	110.5 ± 11.1	156.0 ± 2.1

Significance evaluated against group 4: a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$

observed only 2 brain tumours (2/24 = 8.3 %). The latency times were uniformely within a rather small range between 335 days and 369 days.

3. DISCUSSION

The AT activity of controls is higher in the liver than in the brain. However, AT activity is higher in the rat fetuses than in the adults: in the fetal brains 2.3-fold, in the fetal liver 1.4-fold. Application of either treatment alone or in combined treatments affected AT activity in the brain of both the dams and fetuses in a similar manner. The major finding was a clear decrease in the AT activity after ENU treatment. This correlates with a significant increase in the incidence of brain tumours. The inductive effect of X-irradiation on AT activity corresponds in turn with a reduction of this tumour incidence after the combined treatments.

4. SURVEY

about the complete period of the contract

The aim of the present research field developed from the observation of a reduced response of prenatally X-irradiated brain of rats to a single dose of the carcinogen ethylnitrosourea (ENU) which mainly induces tumours of the brain in the offspring of treated dams. When ENU was applicated alone during the early fetal period (gestation day 13-15), this resulted in about 60-70 % glioma frequency in the offspring within 30 months. X-irradiation (0.5 - 1.5 Gy) before ENU application reduced the brain tumour frequency in a dose-dependent manner.

Our first studies concentrated to the early effects of X-irradiation at the fetal rat forebrain. Even after a short interval of 2 hours the first alterations of the neuroglioblast cell population were observable. They consisted of a change of the glycoprotein compounds of the cytoplasmatic membranes, which was detectable by a newly arising affinity to Dolichos biflorus lectin which specifically binds to N-acetylgalactosamine residues.

4.1. If the early membrane alterations of brain cells are detectable by lectins, why should not be there endolectins within the damaged brains which are binding to the altered cells and thereby initiate reparation processes? This suggestion led us to our studies about growth control by lectins of glial cells, initiated to carcinogenic transformation by ENU, as well as of glioma cells. These studies were done both *in vivo* by early treatment of the rat offspring with lectins, and *in vitro*.

The following results were compiled until now:

a. *In vivo* studies

Daily applications for 5 days of 10 mg/kg Wheat germ agglutinin (WGA) increases brain tumour response after prenatal application of ENU (50 mg/kg) on gestation day 14 (83.6 % versus 56.2 %, respectively; $p < 0.05$). It was suggested that WGA is an antagonist of nerve growth factor (NGF) at its receptor site. NGF ist known for its inhibitory effects upon ENU-induced trigeminal tumours. Thus, the tumour-promoting effects of WGA may be explained. Conversely, the presence of a WGA-like endolectin within prenatally in X-irradiated brains may be not responsible for the combined effects of X-irradiation and ENU, although an increased WGA affinity of X-irradiated cells was described by others.

b. *In vitro* studies

Glioma cells were grown in culture flasks. Addition of Concanavalin A (10 µg/ml), a mannoside-specific lectin resulted in a long-lasting growth depression of the glioma cells. This was evident both by mitotic rate, reduced ^{3}H-thymidine labelling, shape alterations of the cells, and by an increased intracellular content of intermediary filaments (GFAP-immunocytochemistry).

4.2. As we hypothesized the presence of a growth-depressing, soluble and diffusible (lectin-like) mediator acting upon ENU-transformed glioblasts arising in fetal brains subsequent to X-irradiation, we initiated transplantation experiments for its proof.

Mice were X-irradiated on gestation day 14 with a dose of 1.0 Gy. This animal model was chosen, because of a well known pattern of lesions induced by X-irradiation. Newborn were grafted intracerebrally with cells of the C6 glioma line. This procedure resulted in a poor acceptance of the grafts by the prenatally X-irradiated offspring. In contrast, sham-irradiated controls revealed a rapid take of the C6-grafts, getting moribund within 6 days. These differences were markedly and significant. Also by histology a wide spread infiltration of the C6-grafts in X-irradiated brains with leucocytes was a prominent finding. From this, severe necrosis of tumour areas resulted which are correlated with the longer survival time of these animals.

4.3. As an additional growth-modulating mediator in prenatally X-irradiated brains we suggested alterations in the opioid system. We tried to correlate the changes of opioid receptor status of forebrain neuroglioblasts with the well known deficits in histological detectable repair capabilities in

prenatally X-irradiated brains. These experiments were performed upon the hypothesis that enhanced opioid-/and/or opioid receptor density results in suppression of perinatal cell proliferation within the brains, thus preventing reparation processes from any overshooting effect. Consequently we studied the reparation processes of prenatally X-irradiated rats (1.0 Gy on gestation day 14) in response to postnatal daily applications of naltrexone (50 mg/kg) which is a specific endorphin receptor antagonist. This chronic suppression resulted in a two-fold pattern of late effects in the forebrain. The first effect, an early appearing intermittent hyperplasia of the subependymal layer seemed to be beneficial for cell renewal and reparation of the radiation-induced lesions. The second finding was a long-lasting proliferation capacity even of those glioblast cells which are heterotopically dispersed to more peripheral cortical sites. However, the latter proliferation capacity did not look beneficial for the animal, as it showed symptoms of a dysplastic growth, not contributing to efficient reparation or regeneration of the brain.

5. SYNOPSIS

We described both genetic (DNA-related) effects and epigenetic effects after X-irradiation which are directly correlated with the reduced carcinogenic efficacy of a subsequent ENU treatment upon glial cells:

5.1. The DNA-related effects are reduction of alkyltransferase activity after ENU alone, but an augmentation after combined treatments of rat fetuses with X-irradiation and ENU. This corresponds with a decreased tumour response after combined treatments.

5.2. The epigenetic effects which we have studied are an upregulation of the cellular opioid system due to prenatal X-irradiation. This system is represented by various opioid receptors on the cell surface which are part of a communication system, engaged in perinatal growth modulation. Upregulation of the opioid receptors is connected to fetal growth suppression. The cellular proliferation rate, however, is positively linked to the carcinogenic effects of ENU. Therefore the upregulation of the opioid system in fetal brain after X-irradiation stands in direct connection to a reduced tumour frequency after additional ENU treatment.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

Radiology group (Head: Prof. Dr. K. Tempel) at the Institute of Pharmacology, Faculty of Veterinary Medicine, University of Munich, Königinstraße 16, 8000 München 22, West Germany.

V. Publications;

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-C-077-D

Universitätsklinikum Essen
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Title of the research contract:

Investigation into biological dosimetry: Chromosomal damage and teratogenic effects following irradiation of one cell mouse embryos.

List of projects:

1. Investigation into biological dosimetry: Chromosomal damage and teratogenic effects following irradiation of one cell mouse embryos.

Title of the project no.: BI6-C-077-D

Investigation into Biological Dosimetry: Chromosomal Damage and Teratogenic Effects Following Irradiation of One Cell Mouse Embryos

Head(s) of project:

Prof. Dr. C. Streffer

Scientific staff:

Privatdozent Dr. W.-U. Müller, Dr. U. Weißenborn, Dr. S. Pampfer
N. Heckeley

I. Objectives of the project:

Exposure of different cell cycle stages of one cell mouse embryos in vivo and in vitro to different radiation qualities (neutrons, X-rays, beta-rays) and determination of:

1. chromosomal aberrations in the first, second, and third mitosis after irradiation
2. number of micronuclei in the first, second, and third interphase after irradiation
3. teratogenic effects.

Emphasis is laid on the effects of low doses.

II. Objectives for the reporting period:

III. Progress achieved:

INTRODUCTION

Virtually nothing is known on radiation risk of human embryos during preimplantation stage. This is a problem, because women are not aware of a possible pregnancy as long as the embryo did not implant, so that radiation exposure may take place during this time, and nobody has a chance to assess the risk involved in this exposure. Fortunately, development during preimplantation stage is similar in almost all mammals (including man), so that extrapolation from mouse to man will be easier in this case than in many other animal experiments.

In particular, the one-cell stage (zygote) turned out to be very radiosensitive in previous experiments. Studies of one-cell embryos do not only provide us with informations on radiation risk, but in addition offer the chance to look for basic mechanisms of radiation effects. This is made possible mainly by the synchronous development of embryos up to the eight-cell stage, by the high number of different endpoints that can be determined, and by the possibility to carry out in vivo and in vitro experiments.

This report summarizes the results on cytogenetic and teratogenic effects after radiation exposure (X-rays, neutrons, or ß-rays) of one-cell mouse embryos in various cell cycle stages.

METHODOLOGY

All the methods have been described in detail elsewhere. Thus, only a brief outline will be given here. The mouse strain used was "Heiligen-berger Stamm" (a strain similar to NMRI mice).

a) In vitro culture techniques

(Detailed description in: Müller et al. 1985) Three females and one male were mated for 2 hours in the morning (6 to 8 a.m.). After checking for a vaginal plug, pregnant mice were killed by cervical dislocation and one-cell embryos released from the oviduct by cutting

the ampulla. Cumulus cells were removed by a 5 min incubation in hyaluronidase. Embryos were transferred to Kasai's medium (Kasai et al. 1978), supplemented with bovine serum albumin and EDTA. Culture conditions were 37 degrees centigrade, 10% carbon dioxide and 95% humidity.

b) Cytogenetic damage

(Detailed description in: Weißenborn and Streffer 1988a,b) Micronuclei were determined after fixation of the embryos using the technique of Tarkowski (1966). All particles with the following characteristics were counted as micronuclei: a) Stainable with ethidium bromide; b) round-shaped with distinct boundaries; c) diameter between 5 and 20% that of the main nucleus. Chromosome aberrations: Shortly before the mitosis under study, colchicine was injected intraperitoneally (in vivo experiments) or added to the medium (in vitro experiments) and after arresting mitosis in metaphase, embryos were flushed from the oviducts (in vivo studies) or taken out of the medium (in vitro studies). Chromosomes were fixed using the technique of Tarkowski (1966; in vivo studies) or of Mikamo and Kamuguchi (1983; in vitro studies).

c) Teratogenic effects

(Detailed description in: Pampfer and Streffer 1988, Müller et al. 1989) Embryos were irradiated in vivo on day 1 of gestation and the uterus content was checked on day 19 for early and late resorptions, fetal death, living normal or malformed fetuses. Skeletal abnormalities were determined after clearing of the fetuses with potassium hydroxide and staining with alizarin sulfonic acid.

RESULTS AND DISCUSSION

1. Cytogenetic damage

1.1 Exposure to X-rays or neutrons

Table 1 shows the number of structural chromosome aberrations in the first, second, and third mitosis after X-irradiation at different times after conception. In all cases a significant increase was observed in comparison to the control. However, the different times of irradiation revealed different sensitivities: the time of completion of the second meiotic division (1 h p.c.) was the least sensitive period, and sensitivity increased in the order early G2 (9 h p.c.), S (6 h p.c.), G1 (3 h p.c.), and late G2 (12 h p.c.). The comparatively low sensitivity during completion of the second meiotic division may

Table 1: Chromosome aberrations in 1st, 2nd, and 3rd mitosis after X-irradiation (0.94 Gy)

Mitosis	Time of irrad. (h p.c.)	Chromosome type aberrations	Chromatid type aberrations	Total	No. of metaphases
1 to 2	Contr.	0.023	0	0.023	132
	1	0.167	0.034	0.210	60
	3	0.249	0.184	0.433	60
	6	0.124	0.188	0.312	64
	9	0.180	0.140	0.320	50
	12	0.129	0.519	0.648	54
2 to 4	Contr.	0.006	0.036	0.042	168
	1	0.049	0.114	0.163	61
	3	0.139	0.169	0.308	65
	6	0.125	0.145	0.270	96
	9	0.047	0.172	0.219	64
	12	0.176	0.196	0.372	51
4 to 8	Contr.	0.015	0.062	0.077	194
	1	0.086	0.100	0.186	70
	3	0.136	0.269	0.405	74
	6	0.171	0.181	0.352	88
	9	0.106	0.182	0.288	66
	12	0.186	0.228	0.414	70

be due to the peculiar chromatin structure at this stage. The unusually low sensitivity during early G2-phase may depend either on some cells that were still in S-phase 9 h p.c. or on an effective proof-reading by repair systems shortly after DNA-replication. The high frequency of aberrations after exposure in late G2 (12 h p.c.) can be explained by the short time interval available for repair processes before mitosis is starting.

Examination of neutron exposure revealed RBE values between approximately 5 and 7. The best fit for structural chromosome aberrations was a linear plot of the dose response relations irrespective of radiation quality (X-rays or neutrons).

In the second and third mitosis after radiation exposure, a very prominent loss of complete chromosomes was observed. The extent of hypoploidy was independent of the cell cycle stage irradiated, but dependent of radiation dose and quality.

Micronucleus formation (Table 2) shows a radiation sensitivity similar to that one of chromosome aberrations. The frequency of micronuclei

Table 2: Micronucleus formation after X-irradiation (0.94 Gy)

(24 h p.c.: 2-cell stage; 48 h p.c.: mainly 4-cell stage; 54 h p.c.: mainly 8-cell stage).

Time of irradiation (h p.c.)	Micronuclei per cell at 24 h p.c.	48 h p.c.	54 h p.c.
Control	0.013	0.008	0.006
1	0.158	0.066	0.062
3	0.265	0.138	0.079
6	0.209	0.102	0.080
9	0.068	0.048	0.026
12	0.290	0.179	0.169

All the results of exposed embryos (with the exception of the 9 h exposure and 24 h micronucleus determination) were significantly different (P smaller than 0.05) from the control.

was higher than the frequency expected from the sum of acentric fragments and polycentric chromosomes in the corresponding metaphase. After consideration also of the number of lost chromosomes, micronucleus frequency was lower than expected, so that one has to assume, that micronuclei may be the result of the fusion of several chromatin particles (acentric fragments, complete chromosomes).

1.2 Exposure to ß-rays

Table 3 shows the number of chromosome aberrations in the first, second, and third mitosis after a 2hour exposure to ^{3}H-thymidine in G1-, S- or G2-phase. Not unexpectedly, only exposure during S-phase

Table 3: Chromosome aberrations in 1st, 2nd, and 3rd mitosis after exposure to ^{3}H-thymidine for 2 hours in G1, S or G2

Mitosis	Activity (kBq/ml)	Cell cycle phase	Chromosome type aberrations	Chromatid type aberrations	No. of meta-phases	Aberr. per metaphase
1 to 2	Control		2	0	72	0.03
	50	G1	0	0	15	0
	100	G1	1	0	34	0.03
	100	S	0	1	27	0.04
2 to 4	Control		7	1	248	0.03
	100	G1	2	2	97	0.04
	100	S	7	14	86	0.24
	100	G2	1	0	48	0.02
4 to 8	Control		2	1	103	0.03
	100	G1	1	1	63	0.03
	100	S	15	22	73	0.51
	200	S	16	13	24	1.21
	100	G2	1	0	37	0.03

increased the number of chromosome aberrations. Somewhat unexpected, however, is the result that no increase was observed in the first mitosis after exposure. The amount of activity, that was necessary for the induction of aberrations, was much higher compared to ^{3}H-arginine (Table 4).

Table 4: Chromosome aberrations in 1st, 2nd, and 3rd mitosis after exposure to ^{3}H-arginine for 2 hours in G1, S or G2

Mitosis	Activity (kBq/ml)	Cell cycle phase	Chromosome type aberrations	Chromatid type aberrations	No. of meta-phases	Aberr. per metaphase
1 to 2	Control		2	0	72	0.03
	12.5	G1	2	0	65	0.03
	50	G1	0	0	24	0
2 to 4	Control		7	1	248	0.03
	2.5	G1	28	13	86	0.48
	2.5	S	5	13	48	0.38
	2.5	G2	8	6	43	0.33
	12.5	G1	85	31	96	1.21
	12.5	S	16	39	53	1.04
	12.5	G2	23	24	52	0.90
4 to 8	Control		2	1	103	0.03
	2.5	G1	9	6	45	0.33
	2.5	S	9	8	65	0.26
	2.5	G2	11	10	61	0.34
	12.5	G1	13	9	41	0.54
	12.5	S	20	19	58	0.67
	12.5	G2	19	23	59	0.71

Exposure to ^{3}H-arginine showed additional peculiarities. Irrespective of the cell cycle phase exposed, an increase in the frequency of aberrations was observed. This is in line with the fact that histone synthesis in early embryonic development is not restricted to a single cell cycle phase. The ratio of chromosome type to chromatid type aberrations is clearly dependent on the cell cycle phase exposed. This ratio is correlated with the number of disintegrations occurring before or after DNA replication. Again, however, no increase in the frequency of aberrations was observed for the first mitosis after exposure.

These results underline previous results (Müller et al. 1987), in which the high potential of exerting deleterious effects on early

embryonic development has been shown for ^{3}H-thymidine and, in particular, for ^{3}H-arginine.

2. Teratogenic effects

The major radiation risk during the preimplantation period is prenatal death. However, there are some reports in the literature pointing also to an enhanced teratogenic risk for the surviving fetuses. Our results (Table 5) support this conclusion: there was a marked dose-dependent increase in the frequency of gross abnormalities after irradiation of

Table 5: Prenatal death and malformations after irradiation of 1-cell mouse embryos 1 h p.c.

Treatment	Number of live fetuses	Number of live fetuses per mouse	Number of malformed fetuses (percentage)
Controls	216	8.1	2 (0.9)
Neutrons			
0.12 Gy	245	6.6	6 (2.5)
0.25	160	5.7	12* (7.5)
0.50	220	4.2	19* (8.6)
0.75	119	2.4	28* (23.5)
X-rays			
0.25 Gy	133	6.7	3 (2.3)
0.50	197	5.5	6 (3.1)
1.00	147	4.3	10* (6.8)
2.00	178	2.2	35* (19.7)

* Significantly different from control at P smaller than 0.01

1-cell mouse embryos 1 h p.c. with either neutrons or X-rays. The most prominent malformation was gastroschisis, whereas exencephaly was observed at much lower frequency. The number of malformed fetuses increased with a quadratic function of neutron or X-ray dose, whereas fetal death was compatible with a simple exponential function. RBEs were in the range of 2 to 3.

Malformations were not only inducible 1 h p.c. (Table 5), but throughout the first day of gestation (Table 6) and the entire

Table 6: Prenatal death and malformations after X-irradiation of 1-cell embryos.

Treatment	Number of live fetuses	Number of live fetuses per mouse	Number of malformed fetuses (percentage)
Control	216	8.1	2 (0.9)
1 Gy/ 1 h p.c.	147	4.3	10* (6.8)
Control	527	8.0	6 (1.1)
1 Gy/ 3 h p.c.	343	2.9	22* (6.4)
Control	204	7.9	4 (2.0)
1 Gy/ 6 h p.c.	247	4.5	13 (5.3)
Control	200	7.7	6 (3.0)
1 Gy/12 h p.c.	251	5.3	10 (4.0)

* Significantly different from control at P smaller than 0.01

preimplantation period (data not shown). Radiation sensitivity, however, varied considerably, with the early times after conception (1 h p.c., 3 h p.c.) being the most sensitive. It is particularly important, that there was no indication of a threshold dose after exposure of 1-cell embryos.

The study of skeletal malformations revealed that there was no general increase in the number of skeletal abnormalities after X-ray exposure on day 1 of gestation, but that those fetuses that already showed a gastroschisis had more skeletal defects than either the controls or the irradiated fetuses without a gastroschisis.

The results of the teratogenic experiments emphasize that one should not look at the preimplantation period only as a period of either embryonic death or unimpaired development after irradiation. Obviously, there exist conditions under which those embryos that survive irradiation are at an increased teratogenic risk.

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Int. J. Radiat. Biol. 54, 381-394

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The one-cell mouse embryo: cell cycle-dependent radiosensitivity and development of chromosomal anomalies in postradiation cell cycles.
Int. J. Radiat. Biol. 54, 659-674

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

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V. Publications:

1.

Streffer, C.: Risiko nach Strahlenexpositionen während der pränatalen Entwicklung des Menschen.
Strahlenschutz in Forschung und Praxis, Band XXVIII (1987) 34-47

Pampfer, S.; Streffer, C.: Prenatal death and malformations after irradiation of mouse zygotes with neutrons or X-rays.
Teratology 37 (1988) 599-607

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Int. J. Radiat. Biol. 54 (1988) 381-394

Weißenborn, U.; Streffer, C.: The one-cell mouse embryo: cell cycle-dependent radiosensitivity and development of chromosomal anomalies in postradiation cell cycles.
Int. J. Radiat. Biol. 54 (1988) 659-674

Müller, W.-U.; Streffer, C.; Pampfer, S.: Teratogenic effects of ionizing radiation after exposure of preimplantation stages of mouse embryos.
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Taylor&Francis, 1989, 377-381

Weißenborn, U.; Streffer, C.: Analysis of structural and numerical chromosomal aberrations in the first and second mitosis after irradiation of two-cell embryos with X-rays.
Radiation Research 117 (1989) 214-220

Müller, W.-U.; Streffer, C.: Lethal and teratogenic effects after exposure to X-rays at various times of early murine gestation.
Teratology, submitted for publication

2.

Pampfer, S.: Etude comparée des effets délétères des neutrons et des rayons X sur le développement prénatal du zygote de souris irradié in vivo. Université Catholique de Louvain, 1986. (Thesis)

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Poster on the occasion of the 8th International Congress of Radiation Research, Edinburgh, July 1987 (Abstract No. D41-1P, page 195 in Vol. 1 of the Proceedings of this Congress).

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-C-079-NL

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Title of the research contract:

Development of conditions allowing restoration of hemopoiesis by allogenic purified and in vitro multiplied pluripotent hemopoietic stem cells.

List of projects:

1. In vitro multiplication of purified rhesus monkey and human pluripotent hemopoietic stem cells.
2. Non-lethal multi-modality conditioning for transplantation of T-lymphocyte depleted stem cell fractions.

Title of the project no.:

Project I

I. In vitro multiplication of purified rhesus monkey and human pluripotent hemopoietic stem cells.

Head(s) of project:

G. Wagemaker

Scientific staff:

J.J. Wielenga, F. van Gils, H. Burger, V. Smit

I. Objectives of the project:

The project is directed at purification of rhesus monkey and human stem cells and their subsequent in vitro multiplication to achieve sufficient numbers of stem cells for sustained engraftment using partially or completely mismatched donors.

II. Objectives for the reporting period:

The objectives of the project can be summarized as follows:

1. The development of a method allowing for rapid and large-scale purification of rhesus monkey and human hemopoietic stem cells by positive identification.
2. The identification of hemopoietic growth factors which stimulate proliferation of hemopoietic stem cells.
3. The development of a culture method that supports the proliferation of pluripotential hemopoietic stem cells.

III. Progress achieved:

PROGRESS REPORT 1985 - 1989

1. The development of a method allowing for rapid and large-scale purification of rhesus monkey and human hemopoietic stem cells by positive identification.

The purification of rhesus monkey and human bone marrow hemopoietic stem cells was pursued by a combination of phsyical methods (density gradient centrifugation as a pre-enrichment step and E-rosette sedimentation or cytotoxic lysis using the monoclonal antibody Campath-1 as methods to remove T lymphocytes) followed by positive selection of stem cells either on the basis of expression of class II (DR) MHC-antigens or expression of HPCA-1 (CD34) antigens.
The presence of DR-antigens on the rhesus monkey hemopoietic stem cell was shown by measuring the repopulating capacity in an autologous regeneration assay of BM grafts depleted well as enriched for DR-positive cells progenitor cells. The latter were pre-enriched by discontinuous albumin density centrifugation and complement mediated lysis using CAMPATH-1. In the grafts depleted of DR-positive cells, the repopulating capacity was at least 20-fold depleted. In the grafts enriched for DR-positive cells, an at least 40-fold enrichment of the repopulating capacity was measured. Thus, by these two criteria, the repopulating stem cells of the rhesus monkey are DR-positive cells. Unfortunately, the DR-antigen density of the repopulating stem cells appeared to be heterogenous, also DR-dull cells being highly enriched for stem cells. This heterogeneity of DR-expression renders selection of DR-positive cells insufficiently suitable for transplantation procedures, since high yields can only be obtained at the expense of considerable contamination with DR-negative cells.

DISTRIBUTION OF CELLS STAINING WITH ICH3-FITC (ANTI-HPCA1) IN T-LYMPHOCYTE DEPLETED STEM CELL CONCENTRATES

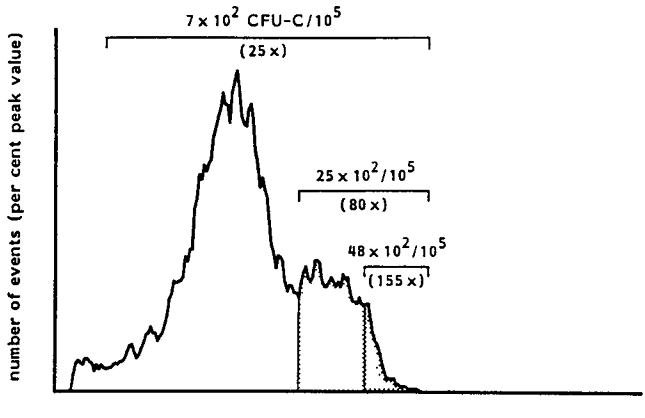

Figure 1. CD34-expression on Rhesus monkey CFU-C, histogram of pre-enriched Rhesus monkey BM cells. The shaded area represents the window set for sorting positive cells. The double-shaded area represents the window set for sorting the 3% most bright cells.

An advance was made when a similar technology was used to assess the potential of the anti-CD34 monoclonal ICH3 (collaboration with Prof. R.J. Levinsky, London), which cross-reacts with rhesus monkey cells (Figure 1). Using fluorescence-activated cell sorting, very high frequencies of hemopoietic progenitor cells, as detected by in vitro assay for colony-forming cells, could be achieved by selecting for CD34 positive cells. Furthermore, using a quantitative regeneration assay involving transplantation of standardized numbers of autologous BM cells in lethally irradiated monkeys, the repopulating capacity of the CD34 positive cells appeared to be enriched to a similar degree as the enrichment obtained for progenitor cells. These results demonstrated that the procedure involving labelling with ICH3 did select for cells capable to reconstitute a hemopoietic system in vivo and also implied that the procedure did not change the engraftment properties of the isolated stem cells.
Since the autologous transplantation procedure in 3 kg rhesus monkeys already stretched the capacity of the cell sorter to prepare BM grafts to its limits, it was decided to develop a method that was more rapid and applicable to the scale suitable for the preparation of stem cell concentrates for BMT in adult human patients. ICH3 was coupled to monosized polystyrene particles (Dynal, Norway), which are smooth surfaced, slightly hydrophilic beads with a uniform magnetic core and a diameter of 4.5 micrometer. Various pilot experiments demonstrated, that this procedure did not enable elution of the bound cells from the beads without unacceptable cell loss. To circumvent this problem, we bound Protein A covalently to the magnetic beads and conjugated the ICH3 to Protein A; cells bound to the antibody can be eluted from the Protein A beads by competitive elution using excess soluble IgG, for which bovine plasma was used. Using this method, approximately 1% of the original nucleated cell count was recovered as rhesus monkey ICH3+ cells and contained on the average 70% of the HPC responding to recombinant GM-CSF, using T cell depleted cryopreserved stem cell concentrates as well as unfractionated fresh BM obtained from rhesus monkeys. Progenitor cell enrichment was about 60- to 100-fold, the fraction of residual T lymphocytes 2-3 logs less than the original BM. Autologous as well as allogeneic transplantation experiments in lethally irradiated monkeys revealed rapid regeneration originating from the enriched cell populations as well as sustained chimerism. It was concluded that selection of HSC by means of ICH3/Protein A beads is a safe, rapid and reliable large-scale method to enrich for stem cells and to remove undesired cells from a BM graft. Its large-scale application for human use is being further developed.

2. The identification of hemopoietic growth factors which stimulate proliferation of hemopoietic stem cells.

In mice, the hemopoietic growth factor interleukin-3 (IL-3) has been strongly implicated in proliferation of hemopoietic stem cells. IL-3 stimulates stem cells to proliferate and to produce committed progenitor cells along all bone marrow derived blood cell lineages. It synergizes in this property with IL-1, IL-6 and GM-CSF, the synergizing mechanisms still subject of further investigations. Of pivotal importance to the development of human stem cell multiplication in vitro, therefore, was the isolation, characterization and production of human IL-3.
We cloned the gene encoding human IL-3 in 1986 by hybridization of radiolabelled mouse IL-3 cDNA with a cDNA library obtained from mRNA of

stimulated human lymphocytes. The human gene was identified by sequence homology with the mouse gene in the 3'-noncoding region, based on highly conserved ATTTA repeat units. The gene, that has five exons, codes for an IL-3 protein of 133 amino acids with two cystein residues which are of importance for its biological activity and two potential N-linked glycosylation sites. The overall homology with mouse IL-3 is very low and, hence, cross-reactivity of biological activity between the two species was not observed. Expression of the human gene in prokaryotic as well as eukaryotic cells yielded a functional protein, that stimulates human multipotential bone marrow cells to produce committed progenitor along a variety of bone marrow blood cell production lineages. The biological activity of human IL-3 on human bone marrow cells is completely analogous to that of mouse IL-3 on mouse bone marrow cells. The lack of protein sequence homology between mouse and human IL-3 was more extreme than that for any of the other known hemopoietic growth factors, which predicted a higher than usual rate of evolutionary divergence for IL-3. The pharmaceutical development of human IL-3 is in progress.

When we tested human IL-3 for its biological activity on rhesus monkey bone marrow cells, we noted a considerably lower effectiveness than observed for human bone marrow cells. We recognized that preclinical studies with rhesus monkeys required identification, characterization and production of rhesus monkey IL-3. Hence, the molecular cloning of the gene encoding rhesus monkey IL-3 was accomplished in 1988 by screening a Lambda Charon 40 library containing Rhesus monkey genomic DNA with human IL-3 cDNA. The nucleotide sequence of the rhesus monkey IL-3 gene compared with the human IL-3 gene displays more than 90% homology and is, similar to the human gene, divided into five small exons; the mature protein comprises 124 amino acids, which is 9 residues shorter than the human counterpart. Overall protein homology is about 80%. The two cystein residues that play an essential role in protein folding in the murine as well as the human IL-3 are also conserved in rhesus IL-3. The rhesus IL-3 gene expressed in COS-cells yielded a product that was biologically active using rhesus as well as human BM cells as targets, although the human IL-3 so far was found to be considerably less active on rhesus monkey BM cells. Expression of the rhesus IL-3 gene in a bacterial species resulted in a highly efficient production, which enabled purification of rhesus IL-3 and its further characterization. Rhesus monkey IL-3 was approximately 100-fold more active than human IL-3 in stimulating rhesus monkey multipotential progenitor cells, but there was no difference in biological activity betwen the two IL-3 species when human bone marrow cells were used as targets. Hence, the encountered species barrier of human IL-3 in rhesus monkeys is unidirectional. Pairwise comparisons of the IL-3 of the coding sequences for mouse, rat, gibbon, rhesus monkey and human IL-3 revealed that IL-3 in general is a rapidly evolving protein, especially human IL-3 evolving from the common ancestral node from which gibbons and humans diverged some 12 million years ago, with the unusual high rate of 2.5×10^{-9} nonsynonymous (i.e. amino acid changing) nucleotide substitutions per site per year. Thus, the unidirectional species specificity of human IL-3 as encountered in rhesus monkeys has been caused by its rapid evolutionary divergence. Although beyond the scope of this report, it is noted that rhesus monkey IL-3 administered to rhesus monkeys stimulated the production of all bone marrow derived blood cell lineages without the need for additional growth factors, thus contrasting results published so far for human IL-3 administered to Macaca species.

3. The development of a culture method that supports the proliferation of pluripotential hemopoietic stem cells.

The recent development of large-scale positive selection of human and rhesus monkey hemopoietic stem cells and the availibility of human and rhesus monkey IL-3 now sets the stage for attempts to multiply purified hemopoietic stem cells in vitro. For mouse stem cells a serum free, chemically defined culture system has been developed, that has been adapted for human and rhesus monkey stem cells. It has been recognized that proliferation of mouse hemopoetic stem cells in vitro is dependent on IL-3 and co-dependent on other hemopoietic growth factors, such as IL-1, IL-6 and GM-CSF, as well as on more generally acting agents, such as hydrocotisone and adrenergic agonists; in addition, stem cells have histamine receptors. As yet, stimulation of proliferation of stem cells results after a few cell divisions in cell differentiation, leading to the production of pathway-restricted progenitor cells and loss of stem cell capacity as tested in transplantation experiments. Thus, although IL-3 has been identified as a primary proliferative stimulus for stem cells, stem cell replication is probably dependent on a complex interplay of stimuli. Our current efforts are directed at testing conditions under which differentiation can be restricted and replication with maintenance of stem cell capacities will be promoted. This fundamental work is performed for human, rhesus monkey and mouse bone marrow.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

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Institute for Experimental Gerontology, P.O.Box 5815, 2280 HV Rijswijk, The Netherlands.
Dept. of Radiobiology, Erasmus University Rotterdam, P.O.Box 1738, 3000 DR Rotterdam.
Dr Daniel den Hoed Cancer Center, P.O.Box 5201, 3008 AE Rotterdam, The Netherlands.

V. Publications:

Included in the publication list of Project II.

Title of the project no.:

II. Nonlethal multimodality conditioning for transplantation of T lymphocyte depleted stem cell fractions

Head(s) of project:

D.W. van Bekkum

Scientific staff:

G. Wagemaker, J.J. Wielenga, F. van Gils, P.J. Heidt, J. Zoetelief

I. Objectives of the project:

This project is devoted to the development of nontoxic (nonlethal) conditioning regimens as an adjuvant to a moderately high doses of total body irradiation, including isolation, chracterization and production of a lymphokine that suppresses the action of T lymphocytes.

II. Objectives for the reporting period:

1. Tests with immunosuppressive monoclonal antibodies as adjuvants to total body irradiation for bone marrow transplantation in mice.
2. Development of a method to purify and produce the lymphokine that suppresses the action of T-lymphocytes.

III. Progress achieved:

1. Tests with immunosuppressive monoclonal antibodies as adjuvants to total body irradiation for bone marrow transplantation in mice.

This project was initiated because it was recognized that acceptance of a MHC mismatched, T-lymphocyte depleted bone marrow graft in primates required a total dose of 12-14 Gy X-rays TBI, which is unacceptable due to lethal side-effects. Therefore, the acceptance of mismatched, T-lymphocyte depleted bone marrow by a recipient requires the development of an immunosuppressive regimen to prevent rejection that lacks the toxicity of such a high dose of TBI. For this purpose, we set out to test possible synergism between TBI and other immunosuppressive agents in mice. A take failure model was developed in F_1 --> P mice to eliminate any influence of immunosuppression caused by graft-versus-host reaction. Initially, agents such as deoxycoformicin, goe 1734, cyclosporin-A and mitoxanthrone were included, but none of these proved to be sufficiently suitable, either due to toxicity additive to that of TBI, or by lack of effectiveness. We therefore shifted to monoclonal antibodies directed against T-lymphocytes.

Monoclonal antibodies directed against T-lymphocytes are thought to be the most promising nontoxic immunosuppressive agents for conditioning for bone marrow transplantation in victims of radiation accidents. The monoclonal antibodies currently tested in mice include rat-anti-mouse L3T4/Lyt-2 (CD4/CD8) monoclonal antibodies of IgG2b isotype (Department of Pathology, Cambridge University, UK) and rat-anti-mouse Thy-1 of IgG2b isotype (Department of Immunology, Institute of Hematology, GSF, Munich, FRG). Currently, we are testing these monoclonals in semi-allogeneic and allogeneic mouse models for partial chimerism, notably alpha-thalassemic mice and W/W^v mice. The former (Wagemaker et al., Transplantation 42, 1986, 248 -251) are an optimal model to test the immunosuppressive potential of monoclonal antibodies, since engraftment can be longitudinally followed in single mice by means of the red cell size marker. Using these mice, it was shown that the anti L3T4/Lyt2 combination of monoclonal antibodies have an immunosuppressive capacity equivalent tot 3 to 4 Gy TBI, while that of anti-Thy-1 is comparable to 2 to 3 Gy TBI. An example of the efficacy of these monoclonal antibodies using mice carrying the thalassemia marker to detect chimerism is shown in Figure 1. It is expected that a further improvement of such a regimen can be achieved by combining these cytotoxic monoclonal antibodies with leukocyte function-blocking antibodies, such as anti-LFA-1. Anti-human LFA-1 was found to cross-react with rhesus monkey cells. Based on the hypothesis that graft rejection has similar effector cells as graft-versus-host reactions, the immunosuppressive effectiveness of anti-LFA-1 was tested in rhesus monkeys in a graft-versus-host model (a fully mismatched donor-recipient combination) as well as a host-versus-graft model (8.5 Gy X-rays as conditioning, using a 3-4 log T lymphocyte depleted, fully mismatched bone marrow graft). Anti-LFA-1 appeared to be incapable to prevent lethal graft-versus-host disease or graft rejection, respectively, and it is concluded that its immunosuppressive action as a single agent is weak. Therefore, the efficacy of such a function-blocking antibody is probably co-dependent on other immunosuppressive agents.

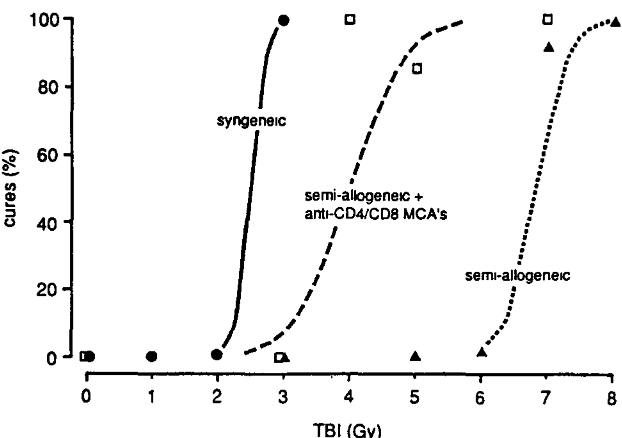

Figure 1. **Efficacy of monoclonal antibodies against T-lymphocytes (L3T4. Lyt-2) in preventing rejection of semi-allogeneic bone marrow, using cure from thalassemia as a marker of chimerism.**

2. Development of a method to purify and produce the lymphokine that suppresses the action of GvH-inducing T-lymphocytes.

The role of suppressor cells in the establishment of transplantation tolerance has been demonstrated by several investigators. It has also been demonstrated that natural suppressor cells, exerting nonspecific suppression are involved in abrogation of T-lymphocyte responses *in vitro* and *in vivo*. The presence of such natural suppressor cells was demonstrated in neonatal spleen and thymus. The natural suppressor cells exert their activity by means of a soluble factor. The culture supernatants of fused mouse neonatal spleen cells and a T cell lymphoma contain a soluble suppressor factor (SUF) that suppresses T-lymphocyte responses both *in vivo* and *in vitro*. In vitro incubation of spleen and bone marrow cells with SUF prior to transfer to lethally irradiated mice, prevents the acute GvHD reaction without affecting the hemopoietic stem cells. Fractionation of the crude supernatant revealed the presence of two suppressive moieties, one of high (>100kD) and one of low (<3kD) molecular weight. These two moieties exert suppression independently and by distinct mechanisms. Only the high molecular weight moiety interferes both with the GvH reaction *in vivo* and with *in vitro* T-lymphocyte proliferation induced by various stimulation signals such as alloantigen, mitogen or OKT3. By studying the kinetics of the high molecular weight SUF moiety mediated suppression it became evident that it interferes at an early stage of T lymphocyte activation. The factor is not species-specific. Its further characterization and production awaits the development of a large-scale purification method followed by molecular approaches.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

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Dept. of Radiobiology, Erasmus University Rotterdam, P.O.Box 1738, 3000 DR Rotterdam, The Netherlands.

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-C-081-B

Centre d'Etude de l'Energie
Nucléaire, CEN/SCK
Rue Charles Lemaire, 1
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Head(s) of research team(s) [name(s) and address(es)]:

Dr. O. Vanderborght
Département de Radiobiologie
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Boeretang 200
B-2400 Mol

Telephone number: 014-31.18.01

Title of the research contract:

Comparison of damage from internal alpha irradiation to the hemopoietic and stromal system in adult and pre- and postnatal animals.

List of projects:

1. Comparison of damage from internal alpha irradiation to the hemopoietic and stromal system in adult and pre- and postnatal animals.

Title of the project no.:

Comparison of damage from internal alpha-irradiation on the hemopoietic and stromal system in adult and pre- and postnatal mice

Head(s) of project:

Greet E.R. Schoeters

Scientific staff:

R. Van Den Heuvel
G. Schoeters
O. Vanderborght

I. Objectives of the project:

This study aims to search for cells at risk with respect to the induction of long-term effects like bone tumors and myeloproliferative disorders. During the latency period before long-term effects appear, effects on stromal and hemopoietic stem cells of hemopoietic organs are studied after contamination of mice with ^{241}Am. The investigation includes different age groups : foetal, neonatal and adult mice. Their sensitivity to internal alpha-emitters will be compared.

II. Objectives for the reporting period:

1 Radiation damage to haemopoietic and stromal stem cell concentration and to long-term bone marrow cultures in which stromal cells maintain haemopoiesis *in vitro*, is investigated after exposure to ^{241}Am *in utero* or injection of adults. The persistence and nature of this damage is analyzed.
2 After contamination of pregnant mice, the transfer of Am to the foetus, its distribution and retention in different organs is analyzed. The absorbed radiation dose is calculated.
3 Study of the stromal cells in haemopoietic liver, spleen and bone marrow during foetal and postnatal development : importance in regulation of haemopoiesis.
4 Initiation of a survival experiment using fostered offspring of Am contaminated pregnant mice : the occurrence of late radiation effects is checked.
5 Development of an *in vitro* assay for osteogenic differentiation of adult bone marrow cells.

III. Progress achieved:

1) Methodology

1.A. Long-term bone marrow cultures are initiated from
a. mice injected with ^{241}Am at adult age (0, 33, 70, 146, 300, 1200 kBq/kg) ;
b. offspring of ^{241}Am contaminated pregnant mice (0, 102, 430, 485 and 1500 kBq/kg) and fostered with a non-contaminated mice ;
c. not-fostered offspring of contaminated pregnant mice (0,480 kBq/kg).
The CFU-GM proliferation is checked in these cultures.

1.B. CFU-s, CFU-GM and CFU-f stem cell assays were performed from the bone marrow of offspring contaminated in utero.

1.C. Co-cultures were performed to analyse which component in the LTC is radiosensitive, either the stromal adherent layer or the haemopoietic cells. Bone marrow cultures depleated from haemopoietic stem cells were obtained via 1) X-irradiation of the LTC or 2) culture conditions in 25% foetal calf serum. Stromal layers were reseeded with haemopoietic cells from control or Am contaminated offspring. CFU-GM numbers in the LTC were followed.

2. Pregnant BALB/c mice were injected with 500 kBq ^{241}Am/kg at the 14 d. of gestation. The Am retention was measured in the offspring between one day after injection until 3 months after birth in liver, skeleton, (femur, calvarium, mandibula, ribs) and gastro-intestinal tract. Ca and Fe content of the selected organs were measured, using an I.C.A.P. emission spectrophotometer.

3. Short-term (CFU-f), long-term stromal (LTC) cultures and co-cultures (between stromal and haemopoietic cell populations of different origin) were established from liver, spleen and bone marrow at different developmental ages.

4. Pregnant BALB/c mice at 14 d of gestation were injected with 0, 100, 500 and 1500 kBq ^{241}Am/kg. At birth, newborn mice were transferred to a fostermother. At 3 weeks of age, mice were separated and housed indidivually.

5. Marrow from femurs of adult mice were collected as a marrow plug and cultured under specific conditions. This osteogenic model is characterized.

2) Results

1.A.a) After ^{241}Am injection of adult mice LTC were less able to support CFU-GM proliferation. The radiation effect lasted at least until 17 weeks after contamination. Damage was observed at the lowest level of injected radioactivity (33.2 kBq/kg). The cumulative dose in the skeleton was then 0.33 gray at 4 w after Am injection.

b) In offspring exposed to Am in utero and reared by a non-contaminated fostermother, stromal bone marrow cells showed no change in their ability to maintain haemopoiesis in LTC. 10 w post-contamination the cumulative dose in the femur reached 0.0098 Gy

and 20 w post-contamination the accumulated dose amounted to 0.0125 Gy.

c) LTC from offspring contaminated in utero and reared by their own contaminated mother, were less able to support CFU-GM proliferation than LTC from non-contaminated offsprings. This radiation damage persisted 71 weeks after contamination. The cumulative dose to the femur was as low as 0.011 Gy.

Although the accumulated doses to the femur were comparable (with 1.A.b.) radiation effects were only seen when Am contamination was a result from the placental transfer and the intake during lactation.

1.B. The haemopoietic stem cell concentrations (CFU-s, CFU-GM) and the stromal stem cell concentrations (CFU-f) in the bone marrow, were not impaired after Am contamination in utero. A diminished stem cell concentration in the initial cell suspension from which LTC were derived can thus not account for the effects seen in the LTC.

1.C. Haemopoietic cells derived from ^{241}Am contaminated offspring had a reduced proliferation capacity on stromal adherent layers which were cultured in 25% FCS. However, when comparing the stroma, the stromal adherent cells derived from contaminated mice supported better the production of CFU-GM.

2. After contamination of pregnant mice with ^{241}Am, 0.02% of the Am is transferred through the placenta to the foetus. In the foetus, Am concentrated in the liver and skeleton. The Am concentration in the skeleton varied considerably between various parts e.g. mandible and calvarium showed a 4 fold higher Am concentration compared to the femur. The amount of Am which initially deposited in a bone depended on the Ca concentration of the bone at that time. The Am retention pattern was different among the considered fetal bones : the Am concentration decreased rapidly in bones with high growth rates.

The fractional retention in the liver remained constant till one month of age. The subsequent decrease occurred simultaneously with a decrease of the Fe content.

Am transfer through lactation resulted in a final increase in Am content by a factor of 3. The incorporation occurred in the skeleton.

The retention data were used to calculate initial dose rates and absorbed radiation doses in function of time in the organs.

3. Studying the changing haemopoietic system during development is useful for the analysis of the role of stromal cells in haemopoiesis which in turn may be important to understand disturbances in radiation induced deregulation of haemopoietic cell proliferation (leukemia). During yolk sac, liver, spleen and bone marrow haemopoiesis in mice, at ages ranging between 11 d of gestation and adult life, important changes in the stromal stem cell population (CFU-f) occur which are correlated with haemopoiesis.

Studies with ^{3}H-thymidine incorporation indicated that stromal stem cells in developing haemopoietic organs have a high proliferation activity (up to 70%) in contrast with adult stromal cells.

In LTC from developing liver, spleen and bone marrow, the proliferation of CFU-GM was supported. The CFU-GM concentrations in the LTC reflected the haemopoietic activity of the organ of origin at the time the culture was initiated. In the stromal layer of the LTC, the presence of

macrophages and glycosaminoglycans (sulphated and non-sulphated) showed a positive correlation with the haemopoietic activity. This correlation did not exist for fibronectin and alkaline phosphatase positive cells. Their presence was inversely related with the proliferation capacity of the stromal stem cells which suggested that these components are a differentiation product of more mature stromal cells.
Co-cultures revealed that the stromal cell compartment in the LTC determined the haemopoietic activity.

4. Results are not completely analyzed yet. Up to now, 403 of 579 offpsring died.

5. Murine adult bone marrow cells exhibit mineralizing capacity in vitro as shown via 85-Sr uptake and morphological studies. The production of extracellular bone matrix molecules was observed in function of incubation time : alkaline phosphatase, osteonectin, osteocalcin and collagen type I. Matrix vesicles and needles of hydroxy-apatite crystals were observed.

3) Discussion

1. Perinatal haemopoietic and stromal cells are radiosensitive and can be considered as target cells for early changes and late effects after contamination in utero. The LTC culture system is a sensitive system to examine radiation damage. Our results indicated that the age of the animals at exposure determined the radiation-induced effects. Supplementary intake of 241-Am via lactation seems important. Using LTC, damage was observed at lower radiation doses if Am contamination occurred at foetal than at adult ages. These observations are important for risk estimation during pregnancy and for neonates.

2. The Am distribution and retention in the foetus (after contamination of the dam) differs from the Am distribution in the adult (after contamination at adult age). This has implications for absorbed dose and risk calculations.

3. The study of changes in the stromal system of haemopoietic organs during development is useful to determine the factors important in the interactions between stromal and haemopoietic cells in regulation of haemopoiesis. Understanding of the mechanisms underlying haemopoiesis can help us to understand the nature of radiation effects.
The large proportion of cycling stromal stem cells in fetal haemopoetic organs, implies that these stromal cells may be very radiosensitive.

4. The in vitro bone formation assay may provide the opportunity to identify the marrow cells with an osteogenic potential (target cells for bone tumor development ?) and to investigate the mechanisms triggering differentiation towards osteogenesis.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

Dr. B. Lord, Radium Holt Institute, Manchester, U.K.
Dr. J. Schmidt, Gesellschaft für Strahlen- und Umweltforschung, Neuherberg, West Germany

V. Publications:

1. Publications in scientific journals

R. Van Den Heuvel
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E. Mathieu
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III D

STRAHLENKARZINOGENESE

RADIATION CARCINOGENESIS

RADIOCANCEROGENESE

RADIATION PROTECTION PROGRAMME
Final Report

Contractor:

Contract no.: BI6-D-064-UK

Medical Research Council
20 Park Crescent
GB-London W1N 4AL

Head(s) of research team(s) [name(s) and address(es)]:

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Title of the research contract:

Studies on myeloid leukaemia and osteosarcoma induced in mice by Ra-224.

List of projects:

1. Ratios of yields of myeloid leukaemia and osteosarcoma induced in mice by Ra-224.
2. The role of oncogene activation in Ra-224 induced myeloid leukaemia.

Title of the project no.: 1

Ratios of yields of myeloid leukaemia and osteosarcoma induced in mice by ^{224}Ra.

Head(s) of project:
E.R.Humphreys

Scientific staff:
E.R.Humphreys

I. Objectives of the project:

To show, in CBA/H mice, that the yield of myeloid leukaemia is greater than that of osteosarcoma following the injection of amounts of ^{224}Ra which are less than optimum for inducing osteosarcoma.

II. Objectives for the reporting period:

1. To continue the investigation of a single injection of different amounts of ^{224}Ra into male CBA/H mice and the identification of subtypes of the induced myeloid leukaemias by cell morphology.

2. To continue the long-term investigation of the effects in male CBA/H mice of the protraction in time of the administration of ^{224}Ra

III. Progress achieved:

INTRODUCTION

The potential contamination of the environment by α-particle emitting radionuclides is a continuing spur to the experimental investigation of their toxicity. Animal experiments (Vaughan 1973, Müller et al 1978) and human experience (Rowland et al 1983, Mays et al 1986) have shown osteosarcoma to be the major late effect. More recently it has been shown that myeloid leukaemia, as well as osteosarcoma, is induced by ^{224}Ra in CBA/H mice and that there may be a range of injected amounts of ^{224}Ra in which more myeloid leukaemia than osteosarcoma is induced (Humphreys et al 1985a, Humphreys et al 1985b).

In most experiments the results from animals have been obtained from single administrations of activity. This contrasts with the continuous contamination which would be likely from a contaminated environment. In a series of long-term experiments carried out at Neuherberg, however, ^{224}Ra was administered to NMRI mice in multiple injections over a period extending up to several months as well as in single administrations. It was shown that ^{224}Ra exerts a much more osteosarcomagenic effect when protracted in time than when given in a single amount (Müller et al 1978). It became clear subsequently, however, that the effect of protracting the administration was reduced when smaller total amounts of ^{224}Ra were administered and at 148Bq g^{-1} and less, the effect was not seen (Müller et al 1983; Muller et al 1989).

In a more recent series of experiments (Müller et al 1988) it has now been shown that significant changes in the yield of lymphoma can be caused by protracting a very much smaller total amount of 18.5Bq g^{-1} ^{224}Ra given to mice over a period of 36 weeks. The mice used in these experiments suffer a spontaneous incidence of lymphoma late in life but the effect of the protracted administration of ^{224}Ra, compared with single injection, is to induce a malignant lymphoblastic lymphoma after a very considerably reduced latent period.

The effect of protracting the administration of α-particle activity has not been comprehensively explored in the CBA/H mouse. In a small experiment in which ^{239}Pu was administered in single or divided amounts it was seen that, for the same total amount administered, more cases of myeloid leukaemia were seen in the animals given ^{239}Pu in divided amounts than were seen in those animals given the same activity in a single amount

(Humphreys et al 1987). The total numbers of leukaemias were small however. The present reports describes experiments which aim to investigate the effect of protracting the administration of ^{224}Ra in CBA/H mice.

The aims of the present ongoing experiments are therefore:

1. To confirm that myeloid leukaemia is induced by single injected amounts of ^{224}Ra which are less than optimum for inducing osteosarcoma.
2. To determine whether, in this range of administered amounts of ^{224}Ra, the yield of myeloid leukaemia is greater than that of osteosarcoma.
3. To determine whether the yield of myeloid leukaemia is increased by injecting the same overall amounts of ^{224}Ra in multiple aliquots over a period of time.

MATERIALS AND METHODS

^{224}Ra was obtained from Amersham Buchler, Braunschweig, GFR via Amersham International, Amersham, United Kingdom as a solution of the chloride in sterile physiological saline. This was diluted with sterile physiological saline containing 100μgcm^{-3} Ca^{2+} (diluting solution - DS) for the single injection experiments to give injection solutions containing 4, 8, 16 or 32kBqg^{-1} and, for the multiple injection experiments, to give injection solutions containing 200, 400 or 800Bq g^{-1}. Duplicate standards were prepared from each injection solution and their gamma-activities determined (using an LKB Compugamma scintillation counter) in a closely-defined region of the gamma energy spectrum which included the 240keV gamma rays from ^{212}Pb.

All of the mice which were used were male CBA/H, 84$\pm$5 days old at first or only injection. In the single injection experiment each mouse was injected intraperitoneally with 0.5cm^3 of the appropriate injection solution; control mice were injected with 0.5cm^3 diluting solution. The mice in the multiple injection experiment were injected intraperitoneally twice each week for eight weeks; they were weighed at the time of injection and injected intraperitoneally with sufficient of the appropriate injection solution to give 32, 64 or 128Bq g^{-1} body mass ^{224}Ra

in total over the eight weeks of the experiment. The control mice in the multiple injection experiment were treated in the same way as the experimental animals except that the injection solution contained no radium.

The animals were not assigned from their litters to their experimental groups in a predetermined order but, because they were assigned at intervals and not all groups were represented at each assignment, the distribution of litters among groups was not entirely random. The animals were housed no more than four to a box (measuring 305mm X 125mm X 120mm) in open accommodation and were allowed unrestricted access to food (Diet 801 157 RM No.1E from Special Diet Services Ltd.) and water. The food was generally in the form of compressed pellets but those mice which developed faulty dentition with increasing age (Humphreys et al 1985b) were given additional powdered diet mixed in a soft paste with water. All animals were inspected at least once each day and a list prepared each week of mice which needed more detailed examination.

The presence of anaemia is the most reliable indicator of leukaemia and was detected most easily in the living animal by pallor of the feet. If anaemia was accompanied by a palpable spleen then leukaemia was more positively indicated. In these animals a tail vein blood sample was taken and blood smears prepared and treated with Leishman's stain. The presence of atypical or immature white cells was most often the signal for the animal to be killed even if the number of white cells seen in the smear did not appear to be increased above the normal range. The mice with suspected leukaemia were weighed and killed under deep chloroform anaesthesia by opening the chest and taking a maximum blood sample from the heart. Further blood smears were prepared and treated with Leishman's stain as above and red and white cells counted in a haemocytometer.

The organs and tissues of the animals were then examined by a standard routine and a record kept of the observations. The spleen was weighed, approximately 100mg homogenised in 5ml Earle's medium and 0.5ml aliquots of the suspension injected intraperitoneally into three 10 - 14 week old female CBA/H mice. Samples of liver, spleen, kidney, at least one lymph node, sternum and lumbar vertebrae were taken from all leukaemic animals and fixed in formol saline before sectioning and staining with haematoxylin and eosin. Samples of other tissues were taken if their appearance warranted closer examination.

Paralysis, particularly of the hind limbs, was sometimes a sign of osteosarcoma in the living animal. These animals and those with palpable masses apparently associated with the skeleton were radiographed under Halothane anaesthesia. Radiographs were taken routinely in two positions - one prone with limbs splayed and the other right side down with limbs in "running" positions. Osteosarcoma was generally inferred from the presence of opacities in the skeleton or less frequently, from the presence of apparent osteolysis. If osteosarcoma was suspected the mouse was killed and given a post-mortem examination as described above but without blood cell counts or transplantation if leukaemia was not also suspected. In these animals samples of the relevant bones were taken for histology in addition to the standard samples from sternum, lumbar vertebrae and soft tissues.

The remaining animals in the experiment were allowed to live until they died or were humanely killed (as above) when they were judged to be able to live for no more than one or two days more or appeared to have a condition which might cause pain or distress. All of these animals were weighed immediately prior to death or, if they were found dead, immediately before the post-mortem examination; they were all X-rayed. Smears were prepared from cardiac blood samples taken from all animals which were killed and samples of organs and tissues were taken from all animals for histology as described above.

Diagnoses of myeloid leukaemia and of osteosarcoma were based on examination of tissues under the microscope.

Myeloid leukaemia does not occur spontaneously in these mice. Any occurrence of myeloid leukaemia, therefore, in mice given ^{224}Ra, was assumed to have been radiation-induced. Similarly, spontaneous osteosarcoma occurs very infrequently and, in ^{224}Ra-treated animals, was assumed to have been induced by the radium.

RESULTS

Tables 1 and 2 show the status of both experiments in January 1990.

Table 1

Occurrence of myeloid leukaemia and osteosarcoma

Status January 1990

Single injection experiment

^{224}Ra injected (Bq g^{-1})	0	69	138	280	550
No. of mice entered	400	400	400	400	400
No. of mice dead	234	281	397	395	231
Mean days inj.-death	620	631	665	669	570
Mice diagnosed as having:					
Myeloid leukaemia	0	4	11	15	11
Osteosarcoma	0	1	3	6	1

Table 2

Occurence of myeloid leukaemia and osteosarcoma

Status January 1990

Multiple injection experiment

^{224}Ra injected (Bq g^{-1})	32	64	128
No. of mice entered	200	200	200
No. of mice dead	52	45	107
Mean days inj.-death	389	358	511
Mice diagnosed as having:			
Myeloid leukaemia	0	0	4
Osteosarcoma	0	0	1

From table 1 it is clear that, in this range of injected amount of ^{224}Ra, more myeloid leukaemia than osteosarcoma is induced. As the experiment nears completion, however, it is likely that more osteosarcoma than myeloid leukaemia will be diagnosed and that the present ratio of myeloid leukaemia:osteosarcoma of 41:11 will decrease.

The investigation of the effect of protraction is at a much earlier

stage and table 2 shows that only four myeloid leukaemias and one osteosarcoma have been diagnosed to date.

DISCUSSION

Mice were not introduced into either experiment at the same rate in all groups. The unequal rates of death and apparent differences in mean period between injection to death are to some extent a reflection of this.

The mean periods between injection and death for the induction of myeloid leukaemia from a single injection of ^{224}Ra were (513 ± 24) days and (485 ± 43) days and for the induction of osteosarcoma (553 ± 64) days and (633 ± 49) days for the present and previous experiments (Humphreys et al 1985a) respectively.

The present experiments suggest that more myeloid leukaemia than osteosarcoma is induced in the range of administered amounts of ^{224}Ra between 70 and 600Bqg^{-1}. It is unlikely, however, that the ratio myeloid leukaemia : osteosarcoma will remain as high as 41:11 as the experiment continues to completion.

If these results are accepted as relevant to humans then persons exposed to amounts of α-particle emitter less than optimum for inducing osteosarcoma may be at risk instead from myeloid leukaemia. There are several features of the myeloid leukaemia seen in these mice which are similar to those seen in human acute myeloid leukaemia and recent (unpublished) observations have shown that of the seven possible subtypes of myeloid leukaemia seen in humans (FAB classification) five have been provisionally identified by blood cell morphology in the mice used in the present experiments. Finally, however, it is only in the elucidation of the mechanisms of leukaemogenesis in both mice and humans that the nature of the this response to radiation can be fully identified and true value of the mouse model understood. Epidemiological evidence from patients treated with ^{224}Ra in the German Federal Republic before 1950 showed conclusively that osteosarcoma was the major late effect (Mays et al 1986). In these studies, although there were 53 cases of osteosarcoma (compared with 0.2 cases expected) there was a stated incidence of only "0 - 3 excess cases of myeloid leukaemia attributable to radium". This evidence appears to show therefore that the sensitivity of humans to α-particle-induced osteosarcoma is at least an order of magnitude greater than is their sensitivity to myeloid leukaemia. The present experiment

shows, however, that in mice there is a region of exposure to ^{224}Ra below that which caused a maximum yield of osteosarcoma in which the yield of myeloid leukaemia is greater than that of osteosarcoma. If these results can be used as a model for man then it might be expected that there is a similar region in human exposure to ^{224}Ra where the incidence of leukaemia may become greater than that of bone tumour. In an epidemiological study of patients given smaller amounts of ^{224}Ra than in the earlier study the three bone tumours which have been diagnosed in the exposed population (fibrosarcoma, reticulum cell sarcoma of the bone marrow and generalized plasmocytoma - Wick et al 1986) contrast with the types of bone tumours (mostly osteosarcomas) which were diagnosed in the population exposed to larger amounts of ^{224}Ra (Mays et al 1986). Leukaemia has also been diagnosed in the patients given the smaller amounts of ^{224}Ra although their induction by radiation has not so far been claimed. The ultimate findings of these studies will have an important bearing on the question of human sensitivity to the induction of leukaemia by bone-seeking α-particle-emitting radionuclides.

To date the multiple injection experiment has not confirmed a general shortening of latent period similar to that seen at Neuherberg in those cases of myeloid leukaemia which have been induced or that a significant increase in the yield of myeloid leukaemia has been caused by protraction. These experiments, however, are still at an early stage.

ACKNOWLEDGEMENTS

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We also thank Diana Robinson for her technical assistance, David Papworth for his careful analysis of the data, Peter Adams for help and advice in taking the X-radiographs, Terry Hacker and his staff for their skills in preparing so much histology and the many members of staff at the Radiobiology Unit who care for the animals.

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Title of the project no.: 2

The role of oncogene activation in 224 Ra-induced myeloid leukaemia

Head(s) of project:

Dr R Cox and Prof G E Adams

Scientific staff:

Dr E R Humphreys

I. Objectives of the project:

To determine the involvement of specific oncogene activation events in the induction of murine myeloid leukaemia (AML) by the bone-seeking radionuclide 224 Ra and whether such activation events are linked with radiation-induced chromosomal changes in target bone marrow cells.

II. Objectives for the reporting period:

1. To use karyotypic analyses to search for consistent chromosomal changes in AMLs 2. To study the status of chromosomal changes in the induction of AML 3. To develop an automated karyotyping system 4. To cytogenetically study the relative leukaemogenic effectiveness of X-rays and α-particles 5. To analyse the structure and expression of proto-oncogenes and growth factor genes in AMLs.

III. Progress achieved:

1 METHODOLOGY

1.1 Karyotypic analysis: The G-banded karyotypes of CBA/H AMLs induced either by 224 Ra or X-rays were analysed both directly (in leukaemic tissues) and indirectly (in short term cultures + or - mitogen stimulation (1,7)). In situ chromosome hybridization of ^{3}H labelled gene probes to metaphase preparations was performed using published techniques (8). The development of a computer-assisted image processing system for the analysis of G-banded murine chromosomes was achieved through close collaboration with the MRC Human Genetics Unit (6,7,9).

1.2 In vitro cellular irradiation and transplantation: Bone-marrow cells were prepared by lavage of the long bone-marrow cavities of male donor mice. Marrow cells were irradiated *in vitro* with 3 Gy 250 Kvp X-rays (cells contained in a medium suspension) or with 1.3 Gy α-particles from a Pu source (cells held as a monolayer on a thin Mylar window). Irradiated and mock-irradiated male donor cells (10^6-10^7) were transplanted by tail vein injection into female recipient mice previously irradiated with 10 Gy X-rays for the purposes of bone-marrow ablation. The clonal proliferation of the irradiated male donor cells was investigated at various post-transplantation intervals by karyotic analyses of bone-marrow, spleen and blood cell preparations of transplanted animals (1).

1.3 Molecular analysis of genes in AMLs: Leukaemic cells were isolated from bone-marrow and spleen of recipient CBA/H mice. High M.Wt. DNA, total RNA and mRNA were purified from cells using standard preparative techniques (12). DNA was digested with restriction endonucleases, electrophoresed on agarose gels, Southern blotted and hybridized to 32P radiolabelled gene probes. RNA analyses were performed by either dot blot or Northern blot techniques. Hybridizing sequences on DNA and RNA blots were detected by autoradiography. For pulsed field gel electrophoresis very high M.Wt. DNA was prepared and

restriction enzyme-digested in agarose plugs. Electrophoresis was performed in a rotating gel apparatus varying voltage and pulse time in order to achieve separation of restriction fragments of M.Wt. 50 kb to 1Mb.

2 RESULTS AND DISCUSSION

2.1 Karyotypic analysis of induced AMLs: A total of 53 leukaemic clones (both primary and passaged neoplasms) were karyotyped; 7 induced by α-particles from 224 Ra, 47 induced by X-rays. Of these, 52 showed some form of chromosome (ch) 2 rearrangement; 36 were scored as deletions, 26 as translocations but unambiguously distinguishing such events in aneuploid neoplasms containing marker chromosomes was not always possible. Since the highest frequency of ch2 translocation was observed in early studies of passaged AMLs that had not been available as primary neoplasms it is also possible that some of these represent clonal selection of further ch2 changes. However, there was no evidence of specific differences between ch2 rearrangement in α-particle and X-ray induced AMLs and sequential karyotypic analysis of passaged AMLs indicated that ch2 change was generally stable. Ch2 translocation in AMLs was in most cases accompanied by loss of chromosomal material. However, although loss of ch2 material between the C and F regions was frequently observed in AMLs this was not invariable and consistent loss of a single ch2 G band could not be determined from these analyses (1,3). Similarly, unlike the situation in human leukaemias that show gene activation events associated with chromosomal change, ch2 breakpoints (BPs) in murine AMLs were not restricted to a single chromosomal sub-region. While the C region (23 BPs) and the E4-F region (26 BPs) were most frequently involved, 6BPs were scored in the centromere - C1 region, 2 BPs in the E1 region and 6 BPs in the G region. Consequently, while it appears that preferential ch2 breakage in radiation-induced AMLs is a feature of the leukaemogenic process, these data do not clearly point towards the consistent involvement of specific chromosomal sites associated with gene activation

events. It is possible, however, that the resolution of G banding techniques are in some cases failing to resolve complex multi-site rearrangements in AMLs (12).

2.2 In vitro cellular irradiation and bone marrow transplantation These studies were initiated in order to determine whether prior 3 Gy X-irradiation of transplanted marrow led to the subsequent repopulation of recipient animals by haemopoietic cell clones carrying specific radiation-induced chromosomal rearrangements. The findings of these studies may be summarised as follows: a) Proliferating donor diploid cell clones carrying stable chromosomal abnormalities were observed in recipient marrow (~ 5 days), in spleen (9-11 days) and subsequently in T-lymphocytes (33-35 days) b) Between 0.3 and 0.5 of such clones carried ch2 rearrangements c) The frequency of such ch2 events (1-1.5 per recipient) exceeded that expected of randomly induced chromosomal damage d) Many ch2 rearranged clones appeared to exhibit some form of preferential recruitment and/or proliferative advantage e) Transplantation of unirradiated cells failed to yield any chromosomally abnormal clones (1). Overall, it was concluded that ch2 events similar to those seen in AMLs were induced directly by X-rays in multipotential haemopoietic cells and appeared to confer phenotypic changes to some carrier cells. Consequently some ch2 rearrangements in AMLs may represent initiating events for this radiogenic neoplasm.

From a total 88 ch2-rearranged haemopoietic cell clones, 54 were scored as deletions, 34 as translocations (usually without losses). However, while as in the case of AMLs, there was clear evidence of ch2 BP clusters in the C and E4-F regions, other regions (B, E1 and G) were also over-represented. Despite their likely relevance to murine leukaemogenesis, no ch2-rearranged transplantation clones have yet been shown to progress to frank AML. The induction by X-rays of ch2 rearrangement in multipotential haemopoietic cells may therefore represent the manifestation of multiple site ch2 fragility following radiation damage thus providing a substantial post-

irradiation pool of genotypically altered cells from which clonal AML only rarely develops.

2.3 Chromosomal rearrangement: Relative effectiveness of X-rays and α-particles: Both human epidemiological and experimental animal studies indicate that α-particles from bone-seeking actinides are only weakly leukaemogenic. This could arise either through intrinsic hypersensitivity of haemopoietic target cells to the lethal effects of α-particles or because the short range of α-particles from bone surface failed to result in the irradiation of a large proportion of these cells. In order to approach this problem experimentally, lavaged marrow cells were irradiated in vitro with α-particles from 238 Pu (1.3 Gy) or X-rays (3 Gy) to give CFU-S surviving fractions of ~ 0.1. Semi-quantitative estimates of the induction of stable chromosomal changes in multipotential haemopoietic cells were obtained by sequential karyotypic analysis of recipient animals receiving α and X-irradiated donor marrow. These analyses showed that whereas X-irradiation induced 3.2 stable chromosomal rearrangements per recipient (~ 50% of which involved ch2) the corresponding figure for α-particles was only 0.25. This result is precisely that which would be expected if target multipotential cells were extremely sensitive to the lethal effects of α-particle traversal and that the majority of cells surviving α-irradiation carry no stable chromosomal damage because they received no α dose to the cell nucleus. Current studies using a partial marrow ablation/self-repopulation experimental strategy for the analysis of in vivo haemopoietic irradiation by X-rays (3 Gy) or α-particles from 224 Ra (16 kBq/mouse) are providing further evidence on the relative inefficiency of α-particles in inducing stable chromosomal changes in surviving multipotential haemopoietic cells. It was provisionally concluded that the weak leukaemogenicity of α-particles from bone-seeking radionuclides may be more strongly influenced by intrinsic target cell radio-sensitivity than by a combination of inhomogeneous α dose and target cell distribution.

2.4 Computer-assisted karyotyping: An image processing system developed for automated analysis of human chromosomes (Piper J and Lundsteen C. Trends. Genet 3 309 1987) was retrained for murine metaphase chromosomes. Initially, 15 CBA/H splenocyte metaphases previously analysed manually were used to train the classifier to identify the centromeric region and the G band pattern of each chromosome. Subsequently, the system was tested for classification accuracy against two independent observers scoring 47 manually selected and 51 automatically selected metaphases. The algorithm for centromeric determination was found to have an error of ~ 9%. Total agreement between the system and the two observers was achieved in ~ 70% of all mitoses. Disagreement between observers on ambiguously banded chromosomes was no less than that between the system and each of the observers. The principal source of system error was distinguishing chromosomes 8 and 12 (7). The mean time for image processing and analysis of a single metaphase was ~ 4 min and since training, ~ 4250 murine metaphase preparations have been successfully analysed. The system has subsequently been trained to analyse Chinese hamster metaphase preparations.

2.5 Molecular analysis of AMLs: Molecular analyses have been principally focussed on the structure and expression of ch2 encoded genes in ch2 rearranged passaged AMLs (2,10,11,12). In these studies normal bone marrow cells were used as controls. No evidence for consistent intragenic rearrangement nor substantial gene expression changes was obtained from analyses of the c-abl, c-src nor β2m loci. Similarly, DNA analyses also failed to show any rearrangements centred on the EMV-15 proviral locus. Negative data on these loci were obtained with up to seven AMLs and it was provisionally concluded that none were specifically associated with ch2 rearrangement. Attention was also paid to an AML (N122) that was characterised by a ch11 → 2 translocation. Murine ch11 contains a conserved chromosomal segment which includes the haemopoietic growth factor (HGF) genes Il-3 and Csfgm; the c-erb proto-oncogene is also encoded on this chromosome. However, DNA and mRNA analyses failed to

provide evidence for rearrangement or changes in the expression of these genes that might be associated with chromosomal translocation. AML N122 did however show significant up-regulation of the normal 5kb c-abl mRNA transcript in the absence of intragenic rearrangement but, to date, N122 is the only AML to show this. HGF genes were also analysed in AMLs N36 (t2:2) and N452 (del 2). With the exception of an intragenic rearrangement and mRNA transcript change in the Csfgm gene of N36, negative results were obtained. In addition, analysis of the proto-oncogenes c-mos (ch10), c-fos (ch12), c-myc (ch15), c-sis (ch15) and c-pim-1 (ch17) also failed to reveal consistent structural or activational changes in up to 7 AMLs. The Csfm (ch3) gene and its receptor encoding gene, c-fms (ch18) were also studied but, again, yielded negative results.

During the course of these analyses the cytokine genes interleukin (Il)-1 α and β were assigned to murine ch2 (D´Eustachio et al Immunogenetics 26, 339, 1987). Using in situ chromosome hybridization techniques Il-1α and β genes were located to the F region of ch2, cytogenetically close to a frequent ch2 breakpoint in AMLs (8). Subsequent in situ analyses revealed that, in the complex t2:2 AML N36, one copy of Il-1β was translocated to the C2 region and appeared to be close to a C2/F translocation junction on the long derivative chromosome. Analysis of mRNA showed evidence of Il-1β but not α gene deregulation in N36 and two other ch2-rearranged AMLs (N122 and N452) but not in a fourth AML (N42A) that carried two cytogenetically normal ch2 copies (12). Conventional Southern analyses showed that Il-1β deregulation in these AMLs was not associated with intragenic rearrangement but that in N36, site-specific demethylation had occurred within the β gene. In order to explore a possible relationship between some forms of ch2 rearrangement and Il-1β deregulation pulsed field gel electrophoresis was used to search for possible rearrangement of Il-1β flanking sequences. These analyses established a preliminary long-range (~ 700 kb) restriction map of the murine Il-1 gene region and showed a) that the α and β sequences are

very closely linked (25-35 kb) within this region and b) that the N36 AML contains a structural rearrangement between 50 and 300 kb from the β gene. The possibility exists therefore that this rearrangement in β gene flanking sequence represents the C2/F translocation junction in AML N36 and that Il-1β deregulation might be directly associated with some forms of radiation-induced ch2 rearrangement in AMLs. This latter possibility was recently shown to be less likely by the absence of Il-1β gene flanking sequence rearrangement in AML N452 which also contains a putative C2/F junction on ch2. Consequently, considerable uncertainty remains on the significance of the Il-1β gene deregulation in the AMLs described here and the flanking sequence rearrangement demonstrated in AML N36.

2.6 Conclusions: Cytogenetic studies have revealed that the ch2 rearrangements and deletions that characterise radiation-induced murine AMLs may have their origins in directly induced chromosomal damage in multipotential haemopoietic cells. Molecular studies have so far failed to unambiguously identify specific ch2 encoded genes involved with ch2 rearrangement but Il-1β appears to be close to a ch2 translocation junction in one AML and may be deregulated as a consequence of a flanking sequence rearrangement. Cytogenetic analysis has been used to show that α-particles are inefficient at inducing stable chromosomal change in multipotential haemopoietic cells and it is suggested that these cells are hypersensitive to the lethal effects of α-irradiation. This may account for the weakly leukaemogenic effects of bone-seeking α-emitting radionuclides. The cytogenetic studies outlined here point towards multi-site fragility of murine ch2 in haemopoietic cells to X-irradiation. The molecular basis of this remains unexplained but some ch2 breakpoint clusters in AMLs and irradiated haemopoietic cells cytogenetically correspond to constitutive chromosomal ´fragile sites´ recently identified in the mouse (Djaladi M et al Hum. Genet. 77, 157, 1987).
Current cytogenetic studies with X-irradiated CBA/H embryo fibroblast cultures suggest that perhaps one copy of ch2 in

this mouse strain is uniquely sensitive to radiation induced breakage and that this unexpected feature is determined by germ line DNA sequences encoding regions of instability possibly imprinted during gametogenesis. If correct, this hypothesis predicts that the high frequency of ch2 rearrangement observed in irradiated haemopoietic cells repopulating marrow-ablated recipients may not reflect any immediate phenotypic effect of ch2 rearrangement (i.e. through gene activation and selective clonal advantage) but is simply a consequence of the extremely high post-irradiation frequency of these events in certain somatic tissues of this mouse strain. This explanation would account for the high sensitivity of a mouse strain, showing a low spontaneous incidence, to radiation myeloid leukaemogenesis. The hypothesis may also imply the requirement for a second genetic event, probably on the other ch2 homologue of a ch2 rearranged/deleted haemopoietic clone, in order to fully initiate leukaemogenesis. Experiments are in progress to further establish these tentative associations between ch2 instability, genetic imprinting and radiation myeloid leukaemogenesis in the CBA/H mouse.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

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Dr A R Shaw, Glaxo IMB 1211, Geneva, Switzerland.

Drs E G Wright and D T Goodhead, MRC Radiobiology Unit,

Chilton, Didcot, Oxon, UK.

V Publications:

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-067-NL

Radiobiological Institute TNO
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Lange Kleiweg, 151
NL-2280 HV Rijswijk

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Title of the research contract:

Relative biological effectiveness for the induction of malignant characteristics in cells by fast neutrons and of lung cancer by radon.

List of projects:

1. Measurements of the biological effectiveness of different types of radiation for induction of chromosome damage and cell reproductive death.
2. Transformation of cells in culture by ionizing radiations of different linear energy transfer.
3. Experimental studies on lung tumour induction by inhalation of radon in combination with some promoting agents, present in cigarette smoke.

Title of the project no.:

Measurements of the biological effectiveness of different types of radiation for induction of chromosome damage and cell reproductive death.

Head(s) of project:

Prof.dr. G.W. Barendsen

Scientific staff:

Prof.dr. G.W. Barendsen, Dr. J. Zoetelief, Dr.ir. H.B. Kal, Prof.dr. J.J. Broerse

I. Objectives of the project:

The objective of this project is to obtain insight in shapes of dose-response relationships and mechanisms by which various types of effects are induced in mammalian cells by ionizing radiations of different linear energy transfer. An analysis with respect to similarities and differences in various parameters of the dose-effect relationships and of RBE values will be performed to assess implications for hypotheses about mechanisms involving the induction of effects by single tracks of particles and by accumulation of damage from different tracks. Possible differences in characteristic parameters are presumably related to differences in repair of damage in cells. Chromosome damage is at least in part the cause of cell reproductive death, but can also cause other changes expressed in surviving cells. A better understanding of these effects will contribute to a basis for assessment of Q-values for radiation protection.

II. Objectives for the reporting period:

Studies were performed to analyze dose-effect relationships for the induction of chromosome damage and cell reproductive death in mammalian cells irradiated with photons and neutrons. To apply modern flow cytometry to the study of radiation induced cellular damage, a high resolution apparatus for flow karyometry has been built, which has been optimalized for the detection of chromosome aberrations.

III. Progress achieved:

Analysis of chromosome damage

In order to obtain insight in the relationships between the induction of chromosome damage and cell reproductive death, experiments have been performed on the scoring of acentric fragments. The ratio's of acentric fragments to dicentrics and centric rings obtained in addition studies were for R-1,M cells: 4.0; for V-79 Chinese hamster cells: 1.8; and for RUC-2 cells: 1.4. The ratio's of cell reproductive death and chromosome aberrations, taking also acentric fragments into account, are equal to 1.3 for R-1,M cells, 1.2 for V-79 Chinese hamster cells and 1.6 for RUC-2 cells. These results indicate that cell reproductive death is associated in a large fraction of the cases with gross chromosome aberrations.

Because of the variable size of acentric fragments and the large amount of labour involved in the scoring of dicentrics, centric rings and fragments, an automated detection method is required to make more rapid progress in this field. For this purpose, a chromosome flow cytometry system has been build which is capable of measuring the DNA contents of individual chromosomes at rates in excess of 1000 chromosomes per second. This system incorporates two high power lasers and a dedicated fast data processing facility. Univariate and bivariate flow karyograms can be measured to assess the frequency of aberrant chromosomes. For these studies chromosome staining with Hoechst 3358 and Chromomicin A3 are employed to detect differences in Adenine-Thymine and Guanine-Cytosine base pair ratio's, respectively. Hoechst 33258 is excited by UV-light and Chromomycin A3 by photons at 458 nm wave length.

V79 Chinese hamster cells were irradiated in G_0 phase with 200 keV X rays and with 14 MeV neutrons. Dose-response curves were determined for three endpoints: I. chromosome aberrations; II. events causing reproductive death and III. chromosome damage detected by flow cytometric analysis of chromosomes isolated from metaphase cells in irradiated cultures. The changes observed in the flow karyotypes from irradiated cultures were quantitatively evaluated by computer analysis.

Cultures were irradiated in plateau-phase containing more than 90 per cent G_0+G_1 cells in order to induce only chromosome type aberrations in metaphase. After irradiation cultures were incubated for 30 min at 37°C, trypsinized, replated in appropriate dilutions, and allowed to start progression through the intermitotic cycle for 16 h.

Measurement of chromosome damage by FCM was performed by methods developed earlier. The technique involves accumulation of cells in mitosis by vinblastine during 5 h, their disruption to prepare a chromosome suspension, measurement of DNA histograms of chromosomes stained with Propidium Iodide and analysis of these histograms by a computer program developed to derive dose-effect relationships.

Cultures used for measurements of visible chromosome aberrations at mitosis were also treated with vinblastine for 5 h. After hypotonic treatment, the cells were fixed on slides and stained with Giemsa. Well spread metaphases were scored for dicentrics, tricentrics, and centric rings.

Cells employed for measurements of clonogenic capacity were cultured for 8 days, fixed, stained with Giemsa and clones containing 50 or more cells were scored.

In the flow karyograms from irradiated cells the heights of the peaks representing intact chromosomes, are reduced and their widths are increased. In addition, the areas between the peaks are partly filled with signals from damaged chromosomes. The extent of the changes observed in the flow karyotypes provides a measure of the radiation effect which at first sight is difficult to evaluate quantitatively.

We employed a method using Fourier analysis to derive a quantitative measure of the radiation damage. The information concerning the shape of the chromosome peaks in the histograms is represented by their Fourier spectrum. Histograms of partly damaged chromosomes show a reduction of the high frequency components of the Fourier spectrum. The decrease in the square root of the sum of the high frequency components of the power spectrum is taken as a measure of the radiation effect observed in the flow karyotypes. By this procedure the increase in background, reduction of peak size and peak broadening all contribute to the final measure of damage.

The frequency of chromosomal lesions registered by changes in the flow karyotype was derived using a computer model. We simulated the effect of breaks in chromosomes, which can cause di- and tri-centrics, a-centric fragments, rings, breaks and deletions. The probability of induction of lesions in each chromosome was assumed to be proportional to its DNA content. Starting from flow karyotypes of unirradiated cultures, series of histograms have been generated demonstrating through this computer simulation the effect of an increasing number of chromosome aberrations per cell on flow karyotypes (Aten et al., 1984). The simulated data were then analyzed by calculating the changes in the Fourier power spectra in the same way as with the flow karyotypes from irradiated cell cultures. By comparing the results of the analysis of the experimental and simulated histograms, we estimated the frequencies of chromosomal lesions in the flow karyotypes from the irradiated cultures.
Comparison of the RBE's for the three endpoints suggests that the FCM technique measures damage which is induced with a similar relative biological effectiveness in dependence on the dose as obtained for impairment of cell reproductive capacity. For the directly visible chromosome aberrations scored, the RBE values in the low dose range are larger.

Analysis of cell reproductive death
Studies of reproductive death of cells cultured *in vitro* have been performed with different cell lines and with neutrons and alpha particles of different energies. In an analysis of these data it was calculated that for cells which had attached to dishes and had spread out to a flat shape, the actual cross section of the cell nuclei perpendicular to the beam of alpha particles can vary, for T-1 human cells used in older experiments as well as for C3H 10 T1/2 mouse cells and NBCH-3 Chinese hamster cells, from values of about 70 μm^2 to values in excess of 300 μm^2. Nevertheless, for irradiations with alpha particles using the tract segment method the cross section for inactivation, derived from own data on published results did not vary greatly. For T-1 human cells the maximum cross section with LET values in excess of 100 keV/μm was 35 μm^2, while for V79 cells and C3H10T1/2 cells values between 30 and 50 μm^2 and for HF human diploid cells a value of about 60 μm^2 have been published. It could be concluded that the probability of inactivation for an alpha particle at the most effective energy of about 2-3 MeV can be described by an effectiveness per unit particle track length through the cell nucleus of between which are all within a relatively narrow range of 0.05 to 0.08 per μm. This would imply that if an alpha particle of this energy passes through a spherical cell with a nuclear volume of 500 μm^3 and a diameter of about 10 μm, the mean track length in the nucleus is 6.3 μm and the probability by a single particle of inactivation could be as large as 0.5, while for a flat shaped cell with a thickness of 2 μm this probability may be as low as 0.1. It can be concluded that no discrepancy exists between data published in the literature about the number of alpha particle tracks through the nuclei required to inactivate mammalian cells, because the observed differences can be ascribed to the time interval between plating and irradiation of cells, which is associated with attachment and spreading of cells on the bottom of culture dishes. In early experiments with T-1 human cells this time interval was only 4 hours and cells had not spread out at the time of irradiation, while in many later studies time intervals of 24 hr between plating and irradiation were used. For an extrapolation to cells irradiated in the human body, e.g. lung cells irradiated by alpha particles from radon daughters, a spherical shape of the nucleus is probably more relevant than a flattened shape.

Composite interaction
From an analysis of data on the relative biological effectiveness of many types of radiation, including heavy particles and neutrons, the suggestion has been derived earlier that for the type of damage causing the single track component, a composite interaction mechanism must be responsible, involving several primary changes. The steep increase of the cross-section versus LET curve between 20 and 100 keV/μm of tissue and the large RBE-values observed, indicate that for the induction of this damage several hundred eV of energy must be deposited in sites with dimensions of the order of 10 nm.
In order to explain what is implied by the suggestion of a composite interaction, it is of interest to hypothesize about the mechanism responsible for the single track type of damage. On the basis of many experimental results concerning the induction of DNA double strand breaks

(DSB), it appears attractive to assume that these breaks are involved in the induction of reproductive death. A DSB is generally assumed to result from two single strand breaks (SSB) close together in the DNA, and therefore constitutes an effect which requires more than an amount of energy equivalent to a single ionization. i.e. more than about 30 eV. However, the most simple hypothesis that a single DSB is responsible for the induction of mammalian cell reproductive death appears to be incorrect. Firstly, a dose of 1 Gy of low- or high-LET radiation causes per cell 20-40 DSB's which evidently are not all lethal, because this dose will cause less than 50 per cent lethality in cells of average sensitivity. It must be concluded that most DSB's do not lead to cell reproductive death but are repaired.

It was concluded (Barendsen, 1988) that an alpha-particle with an LET of 100-200 keV/µm, which impinges on a spherical nucleus of a mammalian cell with 10 pg of DNA, will traverse 50-100 times through a DNA double strand of 2 nm diameter, 20-50 times through a nucleosome of 11 nm diameter or 3-10 times through a chromatin fiber of packed nucleosomes of 30 nm diameter. During such a traversal a large amount of energy is deposited, adequate to induce a composite type of damage. This is consistent with the observation that between 100 and 200 keV/µm the cross section for cell death is constant.

Based on the two types of conclusions from experimental data, i.e., the requirement of a large amount of energy deposited in a small volume with dimensions of the order of 10 nm and the suggestion that high-LET radiations produce unrepairable DNA lesions more effectively, it might be hypothesized that one single DSB is not sufficient for the induction of cell reproductive death but that two DSB's, if produced close together within a distance of the order of 10 nm, constitute the composite effective lesions responsible for cell reproductive death. This would imply that a structure with dimensions larger than the DNA helix of 2 nm diameter is the critical target. Candidates are the nucleosomes with a diameter of 11 nm and the chromatin fibers of packed nucleosomes, with a diameter of about 30 nm.

The hypothesis that lethal lesions induced by single tracks are the result of two DSB's produced sufficiently close together in chromatin fibres, may be extended to suggestions about potentially lethal lesions which might be associated with somewhat larger distances between two DSB's. The distance within which two DSB's have to be produced to cause cell death might be variable and part of the composite lesions might only be potentially lethal, depending on various factors, e.g., on the degree of super-coiling of the DNA and on the metabolic state of the cell. On the basis of this assumption it is possible to interpret differences in radiosensitivity among cells with respect to the initial slope of the survival curve. Only a small fraction of the total number of DSB's is known to be effective, i.e., the number of 20 to 40 DSB's produced by a dose of 1 Gy induces only an average of less than one lethal event. It is well established that almost all of the single strand DNA breaks and a large majority of the DSB's induced in mammalian cells by ionizing radiations are repaired. The composite interaction of two DSB's produced sufficiently close together in the critical structure might prevent their accurate repair and might subsequently result in loss of part of the DNA information. The distance between the two DSB's might be critical, however, with respect to probability that repair mechanisms can eliminate the lesions. Consequently, if this hypothesis is correct, it must be assumed that the initial slope of a survival curve depends strongly on the capacity of cells to repair DSB's.

The hypothesis of a composite DSB interaction would also be consistent with the observation that the RBE values for cell lethality by single events are larger than RBE-values for DSB induction. It is evident that the probability of causing two DSB's close together by a single particle must depend more strongly on the LET than the induction of a single DSB.

A hypothesis about lethal and potentially lethal damage

The hypothesis that lethal lesions induced by single tracks are the result of two DSB's produced sufficiently close together, i.e. within 10 nm, in a chromatin fiber or in two neighbouring fibers, may be extended by suggesting that potentially lethal lesions might be associated with somewhat larger distances between two DSB's produced by single particle tracks. The distance within which two DSB's have to be produced to initiate cell death might be variable and part of these composite DNA lesions might only be potentially lethal, depending on various factors, e.g., on the degree of super-coiling of the DNA, and on the metabolic condition of the cell as

well as its stage in the cell cycle, which may be associated with differences in conformation of the DNA. On the basis of this assumption it is possible to interpret differences in radiosensitivity among cells with respect to the initial slope of the survival curve. These differences might be associated with the degree of packing of DNA in the cell nucleus. This degree of supercoiling may depend on the cell cycle stage and cell conditions. In this way, the paired DSB hypothesis thus provides also the possibility to explain differences in radiosensitivity as a function of cell cycle stage.

The mechanism suggested by the paired DSB hypothesis for cell reproductive death may involve several subsequent pathways. The composite interaction of two DSB's produced sufficiently close together in the critical structure might inhibit repair and could subsequently result in loss of part of the genome. The distance between the two DSB's might be critical, however, with respect to probability that repair mechanisms can eliminate the lesions. Potentially lethal lesions might be assumed to be repairable if culture conditions are favorable, because for these lesions the distance between two DSB's might be larger than for lethal lesions. Consequently, if this hypothesis is correct, it must be assumed that the initial slope of a survival curve, and not only the accumulative type of damage, depends strongly on the capacity of cells to repair DSB's in dependence on their interaction distance.

The hypothesis of a composite interaction of paired DSB's would also be consistent with the observation that the RBE values for cell lethality by single events, as represented by a_1-values in the linear quadratic equation $F(D) = a_1D + a_2D^2$ with D is the dose, are larger than RBE values for DSB induction (Prise et al., 1987). It is evident that the probability of causing two DSB's close together by a single particle must depend more strongly on the LET than the induction of a single DSB. This suggestion would be consistent with the observation by Blöcher (1988) and by Frankenberg-Schwager et al. (1984), that the RBE of α-particles for unrejoined DSB's is increased as compared to the RBE for the total initial double strand breaks.

The hypothesis of a composite DSB interaction can also lead to a suggestion about the dependence of potentially lethal damage on LET. With low-LET particles it is expected that the induction of two DSB's at distances of approximately 10 - 30 nm is a relatively rare event. For most DSB's the distances will be larger and repair is expected to be efficient, preventing lethal damage. Therefore, the effectiveness of a given dose of low-LET particles may be expected to depend strongly on the capacity of cells to repair lesions from two DSB's which are produced at slightly larger distances than required for lethal lesions. Thus, potentially lethal lesions are expected to constitute a relatively large part of the lethal lesions. With increasing LET, PLD is expected to be somewhat more effectively produced, i.e. the RBE increases. But, at very high LET, PLD is expected to be reduced, because the average distance between DSB's becomes smaller than the critical distance of about 10 nm.

It may be concluded that in the context of the hypothesis described, the damage which causes the linear term in the linear quadratic equation, is for low-LET radiation not "unrepairable" damage in the sense that the magnitude of the linear term is independent of the repair capacity of the cell, but rather that it may be strongly dependent on this capacity and, as a consequence, it may vary from cell type to cell type, it may depend on the cell stage in the intermitotic cycle and on cellular conditions during and after exposure. For radiations of very high-LET, these factors are of less importance.

The influence of LET on reproductive death through accumulation of sublethal damage

The damage represented by the quadratic term in the linear quadratic equation, is the type of damage caused by accumulation of lesions. It is known that this damage depends strongly on dose fractionation and dose rate, with repair times in the range of 0.5 - 2 h. Little attention has been given to the analysis of the dependence of this type of damage on the LET. Some information can be obtained from an analysis of the survival curves obtained with α-particles and deuterons (Barendsen, 1967). However, the derivation of a_2-values from these data is associated with considerable uncertainties. Estimates of a_2-values from these curves may have uncertainties as large as 50 per cent. Notwithstanding these large errors, it is evident from the derived data that the cumulative type of damage shows only a small dependence on LET between 5 and 25 keV/μm of tissue (Barendsen, 1989).

This result is consistent with information on the interaction of sublethal damage from doses of high-LET radiation with sublethal damage from X-rays, published by Ngo et al. using fast neutrons, and Bird et al. using α-particles as high-LET radiation (Ngo et al., 1977; Bird et al., 1983). They deduced that at larger doses of high-LET radiation, an interaction was observable, but the amount of sublethal damage produced by high-LET radiation is approximately equal to that produced by the same dose of X-rays. From these results it can be deduced that the RBE for induction of sublethal damage by high-LET radiation is in the range of 1 to 2 and certainly does not increase to a value of 5 - 10 in the range of 20 - 100 keV/μm. It may be concluded that the dependence of sublethal damage on LET is significantly different from the relation obtained for the component of damage which increases linearly with the dose. Nevertheless, sublethal damage does not decrease with LET in the range of 10 - 100 keV/μm and this indicates that it is not associated with simple chemical reactions or SSB's. The differences in RBE-LET relations are consistent with the published data mentiond.
For the induction of sublethal lesions, energy deposition events of the range of 100-150 eV might be sufficient, consistent with the observation mentioned earlier that about 300 eV is effective for inducing lethal lesions, hypothesized to consist of two DSB's. However, whether single DSB's constitute sublethal lesions or whether some other type of DNA damage can interact with DSB's to produce the cumulative type of damage which causes mammalian cell reproductive death, remains to be elucidated. Ward has made the suggestion that locally multiple damaged sites, LMDS, on both strands in a small region of DNA might cause effective lesions (Ward, 1985).

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

Dr. J.A. Aten and Dr. J.B.A. Kipp. Laboratory for Radiobiology, University of Amsterdam

V. Publications:

- G.W. Barendsen. Dose-effect relationships for various responses of mammalian cells to irradiations of different linear energy transfer. J. Soc. Radiol. Prot. **4**, 143, 1984.
- G.W. Barendsen. Comparison of transformation, chromosome aberrations, and reproductive death induced in cultures mammalian cells by neutrons different energies. Radiat. Res. **104**, S158-S164, 1985.
- G.W. Barendsen. Chromosome abnormalities, transformation and reproductive death studied with different radiations and flow karyometry. In: Radiation Research, Proceedings of the 8th Int. Congress of Radiation Research, Edinburgh, July 1987, Vol. 2, 568-574, 1987 (E.M. Fielden, J.F. fowler, J.H. Hendry and D. Scott, eds.). Published by Taylor and Francis, London.
- J.A. Aten, M.W. Kooi, J. Stap, J.B.A. Kipp and G.W. Barendsen. X-ray and neutron-induced chromosome damage detected by flow cytometry compared to cell lethality and chromosome structural changes. Radiat. Res. **110**, 329-339, 1987.
- G.W. Barendsen. Cell survival curve shapes and radiation responses of tumours. The significance of linear term in the linear-quadratic model. In: The Scientific Basis of Modern Radiotherapy. BIR Report 19. (ed.: N.J. McNally). British Institute of Radiobiology, London, pp. 10-17, 1989.

Title of the project no.:

Transformation of cells in culture by ionizing radiations of different linear energy tranfer

Head(s) of project:

Prof.dr. G.W. Barendsen

Scientific staff:

Prof.dr. G.W. Barendsen, Dr.ir. H.B. Kal, and Mr. J.F. Gaiser

I. Objectives of the project:

The research is aimed at obtaining quantitative information and insights in the cellular processes occurring after irradiation which determine the development of malignant tumours. In particular, studies were performed on the relative biological effectiveness of various types of radiations, which differ in the pattern of energy deposition in volumes of sub-cellular dimensions, e.g. chromosomes, because the results can provide information on the mechanisms by which cells are transformed, acquiring malignant characteristics, or are affected with respect to their proliferation and as a consequence have lost the capacity to express altered properties in their progeny. Differences between dose-effect relations obtained with radiations of different linear energy transfer can thus be used to test various hypotheses about carcinogenesis.
Studies on transformation of cells in culture are relevant to carcinogenesis because the clones with altered morphological characteristics can develop into tumours upon inoculation in syngeneic hosts.

II. Objectives for the reporting period:

For the induction of cell transformation it is evident that similarities in dose-effect relationships and the dependence on radiation quality in comparison with the induction of chromosome aberrations and cell reproductive death are observed. But these cannot be considered as proof of a hypothesis that similar primary mechanisms are involved. Cell transformation, which is induced with a relatively low frequency compared to cell reproductive death or gross chromosome aberrations, could in principle be caused by a type of DNA damage which does not represent breakage of a DNA molecule. Therefore, studies on chromosomal aberrations in clones of transformed cells were performed.

III. Progress achieved:

Studies have been carried out on cell transformation *in vitro* by 300 kV X-rays and fast neutrons of 0.5 MeV, 4.2 MeV and 15 MeV energy, using three different cell lines of rodent origin. The aim of these investigations is to compare the dependence of the relative biological effectiveness (RBE) on the linear energy transfer (LET) or linear energy (Y) for cell transformation with similar data for other cellular effects. Such a comparison can yield insights in similarities or differences in the primary stages of the induction of these various types of responses. The comparison of data for three different cell lines is of interest because comparison of data for three different cell lines is of interest because differences in numbers of chromosomes of their DNA content might influence the frequency of the induction of transformation. The cell lines used in our studies differ significantly: C3H/10T1/2 mouse cells with 90 chromosomes, WAG/R-2 rat cells with 42 chromosomes, and NBCH-3 hamster cells with 22 chromosomes.

With the C3H/10T1/2 cells survival curves and dose-effect curves for transformation have been obtained for fast neutrons of 0.5 MeV, 4.2 MeV and 15 MeV energy and for 300 kV X-rays. These curves have been analyzed with respect to values of t_1, t_2, a_1 and a_2 in the representations $F(D) = t_1D+t_2D^2$ for transformation and $S(D)/S(0) = \exp-(a_1D+a_2D^2)$ for survival. The values of the parameters were derived as averages from at least three separate experiments.

The cell survival curves obtained with neutrons of different energies over the range of doses employed, 0-0.4 Gy of 0.5 MeV neutrons and equivalent doses of neutrons of other energies, are not significantly different from exponential curves. For X-rays, doses ranged from 0 to 4 Gy. The curves for cell transformation by fast neutrons are not significantly different from linear curves.

Data for cell reproductive death and transformation of the WAGR-2 and NBCH-3 cell lines have only been measured for 300 kV X-rays and 0.5 MeV neutrons.

The results show that RBE values obtained as ratio's of a_1 values and of t_1 values for neutrons relative to corresponding a_1 or t_1 values for X-rays are very similar for the induction of reproductive death and transformation, respectively. This applies to all three cell lines.

The values of a_1 and t_1 for the different cell lines are presented in Figure 1A as a function of Y_F. The values of Y_F pertain to a sphere of 1 μm diameter. Corresponding data for chromosome aberrations, dicentrics and centric rings, induced in other lines, published by Zoetelief and Barendsen (Int. J. Radiat. Biol., Vol. 43, 1983, p. 319-362), are presented in Figure 1B.

In the present discussion the parameters of the linear terms derived for X-rays will be considered for a comparison with corresponding values for neutrons. The contribution of the quadratic terms for low-LET radiations introduces an increased uncertainty in the estimation of values of the linear terms. As a consequence, the RBE values of neutrons relative to X-rays vary more among different cell types than the dependence of the effectiveness per unit dose on neutron energy. Notwithstanding these uncertainties, the results presented in Figure 1A show that the dependence of the effectiveness for the induction of cell transformation on Y_F is very similar to the corresponding relations for cell reproductive death. A similar conclusion about equal RBE-Y_F relations has been drawn by Zoetelief and Barendsen from a comparison of dicentric and centric ring aberrations with the induction of cell reproductive death in three other cell lines. This is illustrated in Figure 1B.

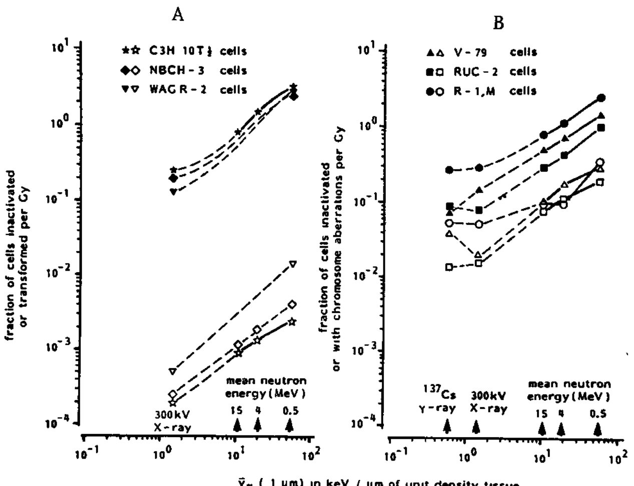

Figure 1: Effectiveness per unit dose as a function of Y_F, the frequency mean of the lineal energy for fast neutrons, derived for effects induced in various types of mammalian cells in culture.

A. Loss of clonogenic capacity (solid symbols) and cell transformation (open symbols), derived from survival curves and dose-effect relations, respectively, for three cell lines. Values of the effectiveness per unit of dose for loss of clonogenic capacity were derived as parameters of linear terms, by analysis of survival curves with the formula: $S(D)/S(0) = exp-(a_1D+a_2D^2)$. Values of the effectiveness per unit dose for cell transformation were derived as parameters t_1 of linear terms, by analysis of the experimental data with the formula $F(D)=t_1D+t_2D^2$.

B. Loss of clonogenic capacity (solid symbols) and induction of dicentrics and centric rings (open symbols), derived from survival curves and dose-effect relations, respectively, for three cell lines. Values of the effectiveness per unit of dose for loss of clonogenic capacity were derived as parameters a_1 of linear terms by analysis of survival curves with the formula: $S(D)/S(0)=exp-(a_1D+a_2D^2)$. Values of the effectiveness per unit dose for chromosomal aberrations were derived as parameters b_1 of linear terms by analysis of the experimental data with the formula $A(D)=b_1D+b_2D^2$.

On the basis of similarities in RBE-LET relations the hypothesis can be advanced that cell transformation, chromosome structural changes and cell lethality are induced by similar primary biophysical events involving large energy deposition events of a few hundred eV in small volumes with dimensions of 10-20 nm causing chromosome structural alterations. However, target regions are larger by a factor of 10^3 for lethal lesions as compared to transforming lesions. Support for this hypothesis was derived form experiments in which in clones from individual transformed cells structural changes of chromosomes were detected by flow karyometry. The NBCH-3 cell lines used for these studies was derived from a newborn Chinese hamster. Between passages 7 and 25 these cells are diploid, contain 22 chromosomes and they can be transformed by radiation. This small number of chromosomes provides an advantage in flow karyotyping as well as in the application of binding techniques. By contrast the 10T1/2 mouse fibroblasts and the 3T3 mouse fibroblasts contain approximately 80 chromosomes.

In previous communications analysis have been presented of karyotypes measured by flow cytometry of cells of an established diploid line of new born Chinese hamster origin, designated NBCH-3. A comparison was made of karyotypes of clones transformed after irradiation of the normal cells, which contain 22 chromosomes. In a bivariate flow karyotype all chromosome numbers 1 to 7 and X and Y can be distinguished, while 8 and 9 coincide.

Clones are scored as phenotypically transformed if the cultures show multilayers and criss-crossing.
Univariate and bivariate flow karyograms showed that the large majority of these clones of transformed cells contained numerical alterations and/or marker chromosomes. The detection of these abnormalities by flow karyometry requires that they are present in the majority of the cells and therefore must have been initiated in the primary irradiated cell or in an early generation of the cells in a clone developing from an irradiated cell. As an initial hypothesis it was assumed that the observed chromosomal changes were correlated with the transformed characteristics of the cells. Inoculation of 10^6 cells of a transformed clone subcutaneously in nude mice yield in 80-90 per cent of the cases sarcomas and the cells are able to grow in 5 per cent agar medium.
Analysis of 25 separate clones did not yield a specific pattern among the chromosomal changes. Alterations occurred in chromosomes number 1, 2, 3, 4, 6, 8 and Y, while in addition marker chromosomes were detected in several clones.
In view of this diversity of karyotypic changes in the clones of transformed cells the hypothesis of a causal relation with the transformed characteristics was questioned. Therefore, it was decided to analyze karyotypes of cells in clones, which developed after irradiation, but which did not exhibit the transformed phenotype. Cells of NBCH-3 passage 19 were plated in five T-25 flasks and irradiated with 4 Gy of gamma rays (137 Caesium). After clone formation, one morphologically transformed and on non-transformed clone was isolated out of each flask and subcultured.
As can be seen in Table 1, both transformed and non-transformed clones contain structural and/or numerical changes of the chromosomes. Extra peaks in the bivariate plots are designated as marker chromosomes (M) without further characterization by G-banding. One clone 1C appears to be normal. However, there are no changes specifically related to the malignant phenotype or to the tumorigenicity. For instance, four clones have lost the Y chromosome. Three clones with malignant phenotype and one with normal phenotype. Out of the three transformed clones, two gave rise to a tumour upon inoculation and one was negative. Clone 1B and clone 2D, though they have a different phenotype, both give rise to a tumour. No other commonly shared aberrations can be detected. From this and previous work, it can be concluded that there is no association of tumorigenicity with a specific chromosomal aberration, as can be detected by flow cytometry. Neither is there an association of transformed phenotype with a specific chromosomal aberration.

Table 1: Summary of characteristics and chromosomal aberrations in irradiated transformed and non-transformed cells.

Clone	Phenotype	Chromosome aberration	Tumour/inoculations
1A	malignant loss of Y	M between chrom. 1 and 2	0/2
2A	normal num. aberr. chrom. 2	M between chrom. 2 and X	0.4
1B	malignant num. aberr. chrom. 4 loss of Y	M between chrom. 2 and 3	4/4
2B	normal	M between chrom. 7 and 8	0/4
1C	malignant	loss of Y	n.d.
2C	normal	no aberrations	0.2
1D	malignant	numb. aberr. chrom. Y	0.4
2D	normal	M between chrom. 2 and 3	3/4
1E	malignant	M clones to chrom. 3	n.d.
2E	normal	loss of Y	0/4

Data on the influence of the dose rate of fast neutrons have been obtained for the NBCH-3 cell line. Dose-effect relationships for transformation frequency per surviving cells were measured for 1 MeV neutrons. It could be concluded that no enhancement of cell transformation is observed for a dose rate of 3 $mGy.min^{-1}$ compared to 150 $mGy.min^{-1}$ with frequencies of 0.8 x 10^{-3} and 1.5 x 10^{-3}, respectively. It should be noted that the uncertainties in these values are about a factor of 2 and the observed difference is not significant.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

Dr. J.A. Aten and Dr. J.B.A. Kipp. Laboratory for Radiobiology. University of Amsterdam.

V. Publications:

- G.W. Barendsen and J.F. Gaiser: Cell transformation *in vitro* by fast neutrons of different energies: implication for mechanisms. Proceedings Ninth Symposium on Microdosimetry, Toulouse, May 20-24, 1985. Radiation Protection Dosimetry, **13**, 145-148,1985.
- G.W. Barendsen: Chromosome abnormalities, transformation and reproductive death studied with different radiations and flow karyometry. In Radiation Research, Proceedings of the 8th Int. Congress of Radiation Research, Edinburgh, July 1987, Vol. 2, 568-574, 1987 (E.M. Fielden, J.F. Fowler, J.H. Hendry and D. Scott, eds.). Published by Taylor and Francis, London.
- J.A. Aten, M.W. Kooi, J. Stap, J.B.A. Kipp and G.W. Barendsen. X-ray and neutron-induced chromosome damage detected by flow cytometry compared to cell lethality and chromosome structural changes. Radiat. Res. **110**, 329-339, 1987.

Title of the project no.:

Experimental studies on lung tumour induction by inhalation of radon in combination with some promoting agents, present in cigarette smoke.

Head(s) of project:

Prof.dr. G.W. Barendsen.

Scientific staff:

Prof.dr. G.W. Barendsen, Dr. B. Hogeweg, Drs. Meijnders, Mr. J.S. Groen.

I. Objectives of the project:

The lung tumour risk for the general public, associated with the inhalation of radon daughters, is generally estimated on the basis of a risk factor, derived from epidemiological data on various exposed groups (e.g. uranium miners, A-bomb survivors and irradiated patients). The value of this risk factor is strongly determined by the data for miners, a group consisting of heavy smokers. From these data, conflicting conclusions, involving protective as well as synergistic action, have been inferred with respect to the risk of the combined exposure to radon and to tobacco smoke. Consequently, no definite risk for a non-smoking population can be estimated.
It is the aim of this study to analyze such interaction mechanisms of combined exposure to radon with other inhaled toxic agents, in order to improve insights in the values of estimates of the radon daughter risk.

II. Objectives for the reporting period:

For the study rats will be exposed to a mixture of radon and formaldehyde and acetaldehyde, respectively. For the last year of the program plans were:
- measurements of the radon daughter concentration levels;
- exposures of WAG/Rij rats to two radon daughter levels of 200 and 800 WLM.

III. Progress achieved:

It is generally assumed that the inhalation of radon at natural background levels, in accordance with the linear dose-effect hypothesis, is associated with a contribution to the lung tumour incidence. On the basis of the current mean radon concentration in Dutch houses of 29 Bq/m^3 and a risk factor for lung tumour induction of 1.10^{-3} cases per Sv lung dose equivalent, it can be estimated that for the Dutch population (during an at risk age between 40 and 80 year) the mean yearly lung tumour incidence associated with this level is approximately 2.1 per 100,000. This corresponds to a contribution to the mean annual death rate from lung tumours for the Dutch population of approximately 8 per cent.
According to Doll (1981), 85 per cent of the lung tumours results from smoking, in particular of cigarettes. Since the risk factor is derived mainly from the epidemiological data on uranium miners, it is relevant for a mixed population consisting of a relatively large percentage (of circa 70%) of generally heavily smoking men. Therefore, it is most likely that the actual contribution form radon inhalation in a general population is much lower than estimated. Consequently, it can be concluded that approximately 10 per cent of the lung tumour incidence must be attributed to exposure to other agents and might result from interactions of combined exposures to radon and the various other indoor agents.
It is the aim of this experimental study on lung tumour induction by inhalation of radon combined with other toxic indoor agents, to analyze these possible synergistic effects. From the epidemiological data of the uranium miners it can be concluded that the lung tumours, associated with the radon daughter inhalation, are mainly of bronchogenic origin. Furthermore, these tumours result from the exposure of the basal bronchial cells to the alpha particles, which are emitted during decay of the radon daughters. Therefore, interactions between this alpha particle radiation and other indoor agents may be expected to occur in this bronchial region. It has been demonstrated (Sterling, 1985), that formaldehyde is a major toxic agent of the indoor air. Since a continuous inhalation of air with a low concentration of formaldehyde may result in irritation and edema of the upper respiratory tract, interactions in the bronchial regions are to be expected from a combined exposure of radon and formaldehyde.
In order to study such interactions in an experimental system, rats will be exposed by inhalation to radon daughters combined with formaldehyde and with acetaldehyde, respectively. Acetaldehyde was selected as second agent on the basis of its occurrence in relatively large concentrations in cigarette smoke. Furthermore, acetaldehyde was selected, in order to differentiate between a cilia toxic and cocarcinogenic action of formaldehyde, since the two agents act differently on the bronchial epithelial cells. On the basis of the lung tumour induction, results in rats derived by Chameaud et al (Chameaud, 1981), it was evaluated that a relatively high level of about 1000 a 2000 Working Level Month (WLM) is required for an optimal detection of a synergistic effect. This is due to the incidence dose curve which demonstrates a linear increase of the incidence up to 3000 WLM and, after reaching a maximum at a level of about 4000 WLM, a decrease with increasing exposure. Such a shape can be explained on the basis of a competition between tumour inducing and cell killing effect of the ionizing radiation. Consequently, above the level of 1000 a 2000 WLM an interference of a synergistic interaction and the cell killing effect can not be excluded. On the other hand, since induction is a stochastic effect and therefore subjected to variations, selection of an exposure level lower than 1000 WLM will decrease the statistical significance.
For the exposure of the rats to this radon level in the mentioned combinations, an inhalation chamber was developed which is based on a "nose only" system. This "nose only" system was selected, since in this system the production of ammonia in the exposure chamber from the action of urea-positive bacteria on urine and faeces can be prevented. The lung tumour induction of radon inhalation, as mentioned, is associated with the exposure of the basal bronchial cells to alpha particles emitted by the various short living radon daughters. Due to their decay properties, concentrations of these daughters are only developed about one hour after the decay of the radon. In order to achieve the required daughter concentrations with a limited amount of radium in the generator, a system as presented in Figure 1 was developed. The total volume of the inhalation chamber is approximately 5 dm^3.

The respired air is, after the passage through a CO_2 absorber via a feedback loop, recirculated in the chamber; the oxygen consumption of the animals is replenished by fresh oxygen, which passes through the radon generator. Detectors and circuits for the monitoring and the regulation of the differential pressures of the various gases (such as radon, O_2 and CO_2) will be connected to the inhalation chamber. As a result of this construction, the activity of the radium in the generator source can be restricted to about 7.5 MBq.

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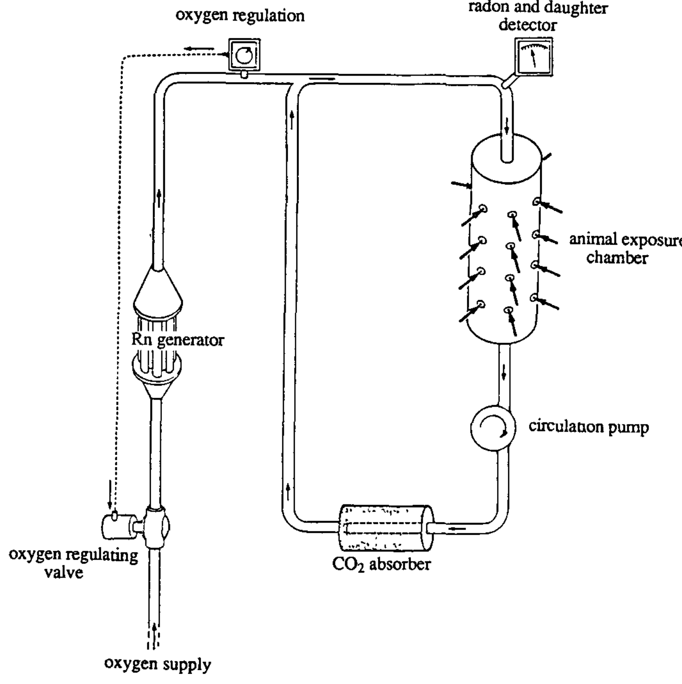

Figure 1: Scheme of "nose only" inhalation system for the exposure of rat lungs to a mixture of radon and aldehydes.

The measurements were performed with the current radon generator, having a radium activity of about 5 MBq.
The efficiency of the radon mixture increases with increasing build-up period, but this corresponds with a low ventilation rate. However, due to the enhanced CO_2 concentration in the

expired air, a low ventilation rate can result in hyperventilation to the animals. Measurements showed that for the constructed "nose only" chamber an air flow of about 11 dm^3 per min is required in order to prevent this hyperventilation. This corresponds to a mean residence time of the radon in the chamber of about 0.5 min. Since the radon daughter concentration is strongly dependent on the residence time, the concentration measurement have been performed for the air flow of 11 dm^3 per min.
For the determination of the radon concentration, a single filter method was selected. After sampling of the air during 5 min with a sampling capacity of 2 dm^3 per min, the activities of the nuclides Po214 and Po218 collected on the filter were measured at different time intervals, using a germanium alpha spectrometer. From these activities, the effective concentration of the daughter mixture can be calculated with the application of the characteristic equations for the decay. Calibration of this spectrometer arrangement with a flat Am241 source, resulted in a counting efficiency of 8.49 per cent for the emitted alpha particles. This value is in good agreement with the calculated efficiency of 8 per cent.
In order to compare the effects of the various delay systems with a system having a continuous air supply, a series of three concentration measurements was performed for this latter condition. This resulted in effective concentrations of 0.05, 0.02 and 0.04 WL.
For the arrangement with the 2 m^3 chamber as delay system, measurements were performed after a build up period of 1 and 16 h, respectively. The effective concentrations were 0.04 and 0.09 WL, respectively. This demonstrates that in comparison to the direct method, even after a build-up period of 16 h, the increase in the concentrations is small. Since radon daughter products will attach to aerosols, smoke was introduced in the air flow of the radon generator in order to measure a potential enhancement. This resulted in an enhancement of the effective concentration by a factor of about 8.
The measurements performed on a system with a small vessel and with no additional smoke aerosols resulted in values for the effective concentration of 0.05 WL and 0.06 WL. The extra addition of smoke aerosols resulted for this system in an enhancement by a factor of about 2 to 3.
Since the application of the smoke aerosols will be unacceptable for the planned inhalation experiments with rats, the effect of water vapour aerosols on the concentration was studied on the system with the small vessels. From these measurements it was concluded that the effectiveness of this vapour is equal to that of the smoke aerosols.
It can be concluded that the resulting effective concentrations with the applied delay systems will be too low in comparison to the required concentration. This is most likely the result of the short periods for build-up of the daughter products. On the basis of the value of about 100 for the ratio of the effectivities for a build-up of 3 h and 30 s, respectively, the more complex recycling system may be expected to approach required concentrations.
The system was completed and tested for the radon concentration at a number of flow rates. A water vapour-aerosol generator was installed in order to increase the probability of attachment of radon daughters to particles in the inhaled air. The efficiency of the radon mixture increases with increasing build-up period, but this is associated with a low ventilation rate. However, due tot he enhanced CO_2 concentration in the expired air, a low ventilation rate did result in hyperventilation of the animals. Measurements showed that for the constructed "nose only" chamber an air flow of about 11 dm^3 per min is required in order to prevent this hyperventilation. This corresponds to a mean residence time of the radon in the chamber of about 0.5 min. Since the radon daughter concentration is strongly dependent on the residence time, the concentration measurement have been performed for the air flow of 11 dm^3 per min.
Radon concentrations were expressed as working levels (WL). The highest level of about 12000 WL at an RaA/RaC ratio of 0.57 was measured for conditions where no rats were present. For other flow rates WL levels were in the range of 4000-10000. However, when rats were placed in the exposure system the concentration decreased greatly to levels of 200-400 WL. It is evident that the inhalation and retention of Radon daughters in the rats greatly influenced the exposure conditions and caused decreases by factors of 10 to 20. Levels between 1000 and 2000 WL were aimed at for further studies.
In order to attain higher exposure levels, collaboration was sought with the Institut de Protection et de Sureté Nationale, France (Dr. Lafuma).

The experiments in Razes, France, and Rijswijk were carried out in 1988 and in 1989 with Rn-222. The WL value can be measured by air sampling, i.e. letting a certain amount of air pass through a membrane filter. the daughters will deposit on the filter and their original concentrations may be derived from measurements of the alpha activity collected on the filter. A well-known method, for measurement of these concentrations has been developed by Tzivoglou et al. (1953) and modified by Thomas (1972). A very rapid determination of the WL number has been developed by Kusnetz (1956), and modified by Rolle (1972).
Ten different concentrations of Rn-daughters the WL value have been determined with both the Rolle and the Thomas method. The results can be found in Table 1. The agreement between the two methods is rather poor. Taking into account their maximum error of 20 per cent (Rolle) and 10 per cent (Thomas), they are allowed to differ up to 25 per cent at the most. Among the six comparisons that can be made, however, only half of them differ between 20 and 25 per cent, while the other three show even greater differences, up to 160 per cent.
Lung burdens have been determined directly by killing rats immediately after a period of 6 hours of Radon inhalation, and measuring the gamma-activity from Pb-214 in the lung as a function of time. The extrapolated activity at t=0 (end of inhalation) is thought to be a reliable measure for the actual dose rate to the lungs. These measurements have been carried out in Razes with the Rijswijk equipment at three different WL values as determined wit the Thomas method.
It was concluded that concentrations of 834 WL in Rijswijk and 3861 WL in Razes result in practically the same deposition of radioactivity in the lung. The same is true for 445 WL in Rijswijk and 1485 WL and 2964 WL in Razes.
The WL values giving the same lung dose are different by a factor of 2 up to 7, the largest discrepancies occurring between WL values determined by different methods. Even if one takes into account the poor agreement between the Rolle and Thomas method, the differences are still large.
We have found that simultaneous measurements of WL values, using the Rolle and the Thomas method, may yield very different results. This leads s to believe that at least one of the two methods should be thoroughly investigated for its accuracy and its applicability in WL measurements.
Furthermore, we found that the WL value as such might be a rather poor measure for purposes of estimating the lung dose. If this can be confirmed, it may have important consequences for the calculations of risk from exposure to Radon and its daughters, both in mining practice and in the field of public health.
We therefore propose that a research project is to be started aimed at clarifying the problems related to WL measurements and the validity of the WL value as a measure for lung dose rates.

Table 1: WL values measured in the Razes inhalation facility

date	Rolle method		Thomas method	
	morning	afternoon	morning	afternoon
11-04-1988	652	665	---	1455
12-04-1988	1747	1858	1435	1485
13-04-1988	1500	1796	3866	---
14-04-1988	2272	2285	2841	2964

The radon chamber for exposure of rats has been completed and exposures of groups of rats to various radon concentrations have been started. Based on the newest data from the scientific research at Razes, France (Dr. Chameaud) it was concluded that the initially proposed exposures to 1000 and 2000 WLM would be too large to study synergism between Radon and acetaldehyde. Consequently, selected levels were 200 and 800 WLM, at which the expected lung cancer incidence would be about 10 and 30 per cent, respectively.

Exposures at these levels have started with 40 rats per group, either radon alone, acetaldehyde alone, and combined exposures to both agents, as well as appropriate sham treated controls. These exposures are still continuing, 50 per cent being completed by December 1988. Results on lung tumour development can be expected to be obtained in 1990.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

V. Publications:

- L.W. Put, R.J. de Meijer and B. Hogeweg. Survey of radon concentrations in Dutch dwellings. Proc. of Exposure to Enhanced Natural Radiation and its Regulatory Implications, Maastricht, 25-27 March, 1985.
- B. Hogeweg. Een analyse van de risicofactoren voor blootstelling van de long aan lage stralingsdoses. RBI-rapport no. 3375, 1986.
- B. Hogeweg. Eindrapportage en evaluatie van het SAWORA-onderzoekprogramma naar het achtergrondniveau van de natuurlijke straling in Nederland. Rapportnr. 3477. Radiobiologisch Instituut TNO, Rijswijk, 1986.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-070-I

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Title of the research contract:

Radiation carcinogenesis.

List of projects:

1. Follow-up of cancer patients treated by radiotherapy for the appearance of a second independent tumour.

Title of the project no.: BI 6 D 070 J

Radiation Carcinogenesis: Follow up of cancer patients treated by radiotherapy for the appearance of a second independent tumor

Head(s) of project: Prof. Aldo Becciolini

Scientific staff: Cellai E.; Balzi M.; Pacini P.; Santoni R.; Cionini L.; Porciani S.; Olmi P.; Chiavacci A.; Biti G.P.; Zanieri E.; De Maria D. (MO); Falchi A.M. (MO); Giachè V.; Lanini A.; Mungai V.; Mungai R.; Pupi A.; Cintolesi V.; Magrini S.; Fallai C.

I. Objectives of the project:

The aim of the study was to evaluate the carcinogenic effect of ionizing radiation in patients with neoplasia in different sites and treated by radiotherapy. The incidence of second primary tumors, histologically proven, was analyzed according to the time elapsed from irradiation and to the appearance in the irradiated volume. The research needs to be completed in order to have a higher number of cases and a larger follow-up. In this part of the study we organized and examined the clinical records of patients treated by radiotherapy in the past 15-20 years or more. The study was carried out in Florence at the University and Hospital Radiotherapy Sections and at the Nuclear Medicine Section for the 131J treatment in the thyroid carcinomas. Also the Radiotherapy Section of the University of Modena participated in the study. The study also examined biochemical, morphological, and cell kinetics parameters that can be used as markers of the radiation damage. These elements could point out correlations with the possible appearance of a second tumor and contribute to the explanation of radioinduced carcinogenesis mechanisms.

II. Objectives for the reporting period:

The study of the past year mainly concerned the analysis of patients with breast carcinoma and treated by ionizing radiations. The incidence of second primary tumors was compared with that of patients undergoing chemotherapy alone. The analysis of patients affected by gynecologic and head neck tumors is still in progress and includes also the cases of the University of Modena.

III. Progress achieved

The possibility that ionizing radiations produce tumors is well documented, but the mechanisms producing the process are not yet known. Radiation carcinogenesis is a stochastic process and its probability increases as the dose increases. However, following very high doses, tumor incidence in the irradiated volume appears to decrease because cell death is more probable than cell transformation induced by the ionizing radiations.

Doses received during radiodiagnostic or nuclear medicine examinations are too low to produce a significant increase in the cancer incidence when compared to the natural incidence. Instead, during radiotherapy an elevated dose is absorbed in the directly irradiated volume while a slightly elevated dose, yet one capable of increasing the incidence of a second primary tumor, is absorbed in the surrounding zone and in the other parts of the body.

In those patients treated with radiotherapy, it is difficult to attribute a second primary tumor to ionizing radiations since it could be the result of a certain spontaneous predisposition to malignant transformations. However, this type of relationship has not been proven.

According to Warren and Gates, all second we considered tumors have a malignant histologic pattern, are not metastases of the first tumor, and must be clearly distinguishable without reasonable doubt from the first tumor.

A continuous, accurate follow up is essential for this type of study. For many years in the Radiotherapy Center (University and Hospital) of Florence many thousands of patients affected by tumors in different organs and tissues were treated with ionizing radiation while others underwent chemotherapy.

Most of this study examined patients with primary tumors treated solely with curative radiation therapy. All cases were histologically proven to be cancer. Patients with metastases previously treated with radiotherapy were excluded. About 2500 patients per year were followed for many years with periodic evaluations of the disease and of possible late radiation damage. About 1800 new patients underwent radiotherapy each year in the Center.

Different radiation sources (2 telecobalt units, Betatron, 2 linear accelerators, after-loading sources) were used for external and endocavity radiotherapy. Moreover, radiometabolic treatments with 131J for thyroid carcinoma, hyperthyroidism and toxic adenoma were performed. Conventional fractionation (2 Gy/die, 5 days/week) was used for external radiotherapy with gamma rays, X rays or accelerated electron beams. More recently, different fractionation regimens (1 Gy x 3/die, 2Gy x 2/die, 2 Gy x 3/die) were introduced for treatment of head and neck cancers. The use of these different radiation sources and treatment schedules allowed evaluation of the different acute and mainly late effects of ionizing radiations.

The aim of the research was to continue the study of patients during follow up to evaluate the incidence of a second primary

tumor in the irradiated area or outside of it. Most subjects were affected by breast cancer, gynecologic tumor, Hodgkin's lymphoma, head and neck (ENT) tumor or thyroid carcinoma. During treatment and follow up, besides clinical and radiological examinations, the concentration of some molecules considered to be important biochemical indicators of radiation injury or tumor markers were assayed to evaluate the correlation of these parameters with the condition of the patient, prognosis, recurrence and the appearance of a second independent tumor.

Particular attention was paid to those patients affected by Hodgkin's disease since there has been increased recovery and a prolonged survival time over the past decades due to improved therapeutic treatment. A total of 918 patients were treated from 1955 to 1985. In two cases (0.2%) the second tumor preceded Hodgkin's disease whereas in 0.4% of patients a synchronous second tumor appeared. In 35 cases (3.8%) the second primary tumor was metachronous. The mean latent period between the first and the second primary tumor was 8.4 years (range 1-21).
The treatment modalities were determined according to the clinical characteristics and the hystopathology of the disease. The second neoplasia was classified in two groups:
a) acute leukemia and non Hodgkin's lymphoma;
b) solid tumor.
To analyze the incidence of a second primary tumor patients were divided into the following three groups according to the type of treatment:
a) 347 radiotherapy only (RT);
b) 482 radio- and chemotherapy (RT+CT);
c) 89 chemotherapy only (CT).
The incidence of a second primary tumor was 5.4%, 2.6% and 3.3% respectively for groups a, b, and c.
Solid tumor in the irradiated volume appeared in 13 cases treated with RT and in 5 cases treated with RT+CT. Six patients treated with RT and 2 treated with RT+CT showed a second tumor outside the irradiated volume. Two second tumors occurred in patients treated with CT. Seven patients contracted leukemia; six of them after RT+CT.

In the 1311 cases of ENT tumors treated with RT there was an incidence of 52 metachronous second tumors. The distribution of the second neoplasia as a function of the location of the first tumor was: nasopharynx 10/223, oropharynx 11/282, hypopharynx 2/80, oral cavity 7/229, larynx 22/368, salivary glands 0/60 and paranasal sinuses 0/69. Most of the second tumors (41) appeared outside the ENT sites. Eight of 11 cases in the ENT region occurred in the irradiated volume. The mean latent period between the first and second tumor was 4.5 years (range 1-12).

Our most recent study primarily examined the incidence of breast tumors. Only the patients treated with postoperatory RT after radical surgery of the primary tumor were included;

radiotherapy has been associated with chemotherapy only in a small number of cases in recent years. Conventional fractionation (2 Gy/die, 5 days/wk for a total dose of 44-50 Gy) was used in all cases. The survey was divided into three periods of observation:
a) 447 cases treated from 1971 to 1975. One patient had already been affected with a tumor and three others had synchronous tumors. Second tumors appeared in 19 patients: 3 cases of leukemia were reported from 4 to 7 years after treatment whereas the others were solid tumors of the stomach (4), lungs (2), K corporis (2), colon (2), ovary (2) and in other places (4).
b) 238 cases treated from 1976 to 1983. One patient had a synchronous tumor and two had a second tumor 5 years after treatment.
c) 706 cases treated from 1984 to 1988. 3 tumors preceded treatment, 2 were synchronous and 2 metasynchronous. Of these 1 case of leukemia appeared after 3.5 years and 1 case of K corporis after 4.5 years.
Out of a total of 1391 cases treated from 1971 to 1988, 23 second tumors were observed in the period following RT (1.64%). However, for many of these the follow up period was short, especially for the appearance of solid tumors, and other clinical records must still be analyzed.
The patients who had relapses or metastases during the follow up were later treated with CT.
The study compared the incidence of patients undergoing RT with a homogeneous group according to age, clinical state, histotype, etc. of patients with breast tumors treated with CT. Out of a total of 2032 patients 3 tumors before breast cancer and 4 synchronous tumors were observed.
In the group of 229 patients treated with CT between 1971-1975 7 second tumors appeared, 4 within 3 years after treatment. In the 1976-1983 period, out of 1250 patients 25 second tumors were observed; of these 1 was leukemia, 13 stomach or colon tumors and 8 ovaric or K corporis tumors. In the 553 patients treated most recently (1984-1988) 3 second tumors have been observed till now.
The overall incidence of second tumors was 1.72%, similar to that found in cases treated with RT. The incidence of leukemia in the 2 groups was different: 4 cases (0.29%) were observed in the group treated with RT compared to 1 case (0.05%) in the group treated with CT. However, too few cases have been observed to make this datum meaningful. Another difference between the two groups concerned the incidence of second tumors found in the gastrointestinal tract: 0.57% in the group treated with RT and 0.84% in that treated with CT. No significant differences were found in the incidence of gynecological tumors (0.43% and 0.49%). It should be noted that in the group treated with RT the second tumor generally had a longer period of latency.

The results of the study of patients with cervical carcinomas concerned primarily cases in advanced clinical stages. According to the clinical stage, patients were treated with RT

and surgery or only RT for the more advanced forms. Patients in stage IIb and with small sized tumors were treated with intracavity RT; the others were treated with external RT. The patients in this latter group received over the pelvis 2 Gy/die, 5 days/week a total dose ranging from 50 to 70 Gy. The clinical records of 577 patients were analyzed, and 12 second tumors were observed. Five of these occurred before cervical carcinoma and 2 were synchronous. In 5 patients metachronous second tumors were observed outside the irradiated volume. The follow up period for this pathology was and is, however, too short.

Part of the study focused on 457 patients affected by thyroid carcinoma and treated with 131J (2.8-7.4 GBq) from 1977 to 1987. Of these patients, 39 were lost during follow up and 25 died of thyroid carcinoma or non neoplastic disease. Four second tumors, located in the breast, kidney, bladder or prostate, were observed during the 2-6 year period after radiometabolic therapy. One case of A.L.L. was observed in a patient who had received 2 doses of 131J (2.8 and 5.5 GBq respectively) with a 19 month interval between doses. The A.L.L. appeared 7.5 years after the second administration.

The Institute of Radiotherapy of the University of Modena recently joined the study on Radiation Carcinogenesis in patients undergoing radiotherapy. A preliminary analysis of the incidence gathered between 1972 and 1987 demonstrated that out of 25000 patients examined 383 had multiple primary tumors: 86 were synchronous and 297 metachronous. A total of 8385 patients who underwent RT were analyzed: all were treated with conventional fractionation (total dose 45-55 Gy) over different parts of the body. During follow up 217 cases of second primary tumor were observed; of these 16 patients (0.19%) had developed the second tumor in the irradiated volume, with an average latent period of 17.7 years (range 8-30). The subdivision of these patients according to the irradiated zone showed that out of 2127 patients irradiated on the pelvis for tumors of the uterus, of the rectum or of the bladder, 4 (0.19%) developed a second tumor of the rectum, 3 (0.14%) of the endometrium and 1 (0.05%) of the ovary. Of the 2255 patients treated in the ENT region 3 (0.13%) developed a second tumor of the larynx or at the base of the tongue. Three second tumors (breast, skin and lung), equal to 0.25%, were observed in 1169 patients treated on the thoracic region; 2 other second tumors of the skin (0.07%) were observed in 2834 patients treated regionally for surgically removed breast cancers.

Simultaneously with the epidemiologic studies other studies were conducted on the patients during treatment and follow up in order to find molecules or biochemical indicators of acute and late damage in the tissues of the irradiated volume; changes in these molecules during radiotherapy could be used to evaluate the effects of increasing doses of ionizing radiations on specific parts of the body. The information

obtained would also be able to be used in the quantitative evaluation of damage after accidental exposure.

The presence of a close correlation between the cell proliferation and differentiation processes and the concentration of the polyamines spermidine (spd) and spermine (spm) persuaded us to study the behavior of these molecules in different tissues and in some body liquids following irradiation.

Preliminary experimental results were obtained from tissues with different proliferative activity (small intestine, spleen, kidneys) from rats exposed to whole body irradiation with 3 Gy (gamma rays from 60Co) and sacrificed between a few hours and 2 months after irradiation.

Verification of the proliferative activity, evaluated by administration of 3H-thymidine 1 hour before sacrifice, demonstrated an optimum correlation between the reduction of the tissue polyamines and the block of the proliferative activity in the days following irradiation. Even the following phase of repair was well represented by an increase of the polyamines in correspondance with the increase of 3H-thymidine uptake.

In the clinical studies, notable modifications of the extracellular polyamines were observed in their urine excretions during the radiotherapy of patients affected by head and neck tumors or uterine cervix tumors. However, the results were highly variable, probably because of both the sample collection and preservation methods and the enzymatic reactions that partially change the meaning of the urine concentrations. For these reasons a study of the polyamines in the red blood cells was begun; this also gave more reliable information because kidney catabolism of the polyamines is avoided. Preliminary results on patients irradiated in the pelvic area showed a significantly reduction of the polyamines; its compatibility with radiation injury is now being verified.

Among the biochemical indicators of damage on specific tissues, the serum tissue polypeptide antigen (TPA) and α-amylase had an optimal correlation with the amount of damage induced in the salivary glands after the first day of treatment in patients with head and neck tumors treated with different fractionation regimens (2 Gy x 1/die, 1 Gy x 3 /die, 2 Gy x 2/die, 2 Gy x 3/die, with a total dose of 60-66 Gy in the first three and 52 Gy in the last regimen). The results showed that α-amylase activity and TPA increased as a function of the total daily dose and that the higher the dose the less the length of the increase: on the first day the values of serum TPA and α-amylase activity showed a correlation coefficient of 0.971 and 0.999 respectively with the total dose. This relationship was still present the second day of treatment but afterwards the increase disappeared.

These results demonstrated that the two molecules were valid indicators of the amount of radiation damage; they were proposed as biochemical dosimeters of exposure, even accidental, for the head and neck region.

Besides the preceding molecules, other parameters were studied during our research. Among these some serum oligoelements (Cu, Zn and Mg) and the acute phase reactant proteins were assayed. During therapy large changes were observed in the concentration of both the olygoelements and the proteins. These changes were only slightly correlated with the effects of therapy and a clear dose-effect relationship did not emerge.
A study is currently being performed on patients irradiated in the pelvic region for treatment of prostate carcinomas. The red blood cells polyamines and prostatic acid phosphatase (PAP), prostatic-specific antigen (PSA) and serum TPA were determined at preestablished intervals during treatment and follow up. Preliminary results seem to indicate dependency between the variations of the concentration of these molecules and the effect of the ionizing radiations.

CONCLUSIONS

The results of the study appear interesting and increase the previously available informations. Since the observation period is still short, especially with regards to the appearance of solid tumors, the study has continued with the characterization of available control groups from the tumor registries. The organization and collection of clinical records performed during this period makes periodic data revision easier for follow up updating. Analysis of the data related to the single neoplastic pathologies must still be completed in the various Centers collected in the research.
In the near future, besides updating the follow up and completion of the study of other clinical records, an epidemiological analysis will be performed in order to evaluate the incidence of second tumors in patients treated with RT as compared to those treated with CT. Furthermore, based on the analysis of the tumor registries, the significance of the increase in the incidence of second tumors in patients treated with RT will be determined.

V. Publications

A. BECCIOLINI, S. PORCIANI, A. LANINI, A. CHIAVACCI, E. CELLAI

Effects of conventional and multiple fractionation treatments on the serum amylase activity

Acta Oncol. 26, 139-142, 1987

A. BECCIOLINI, M.S. TOMMASI, S. PORCIANI, E. FANTAPPIE', E. CELLAI, A. CHIAVACCI

Serum tissue polypeptide antigen (TPA): marker of acute injury on salivary glands during radiation therapy

Int. J. Radiat. Oncol. Biol. Phys. 13, 1339-1342, 1987

A. BECCIOLINI, S. PORCIANI, A. LANINI, M. ATTANASIO

Polyamines in the small intestine of rats after whole body irradiation

Int. J. Radiat. Biol. 56, 67-73, 1989

S. PORCIANI, M. BALZI, A. LANINI, E. ZANIERI, A. BECCIOLINI

Different approaches in detecting the proliferative activity of rat spleen after irradiation

Int. J. Radiat. Biol. 51, 753-754, 1987

M. BALZI, E. SCUBLA, M.B. NINU, O. FINI STORCHI, E. ALAJMO, P. BOANINI, E. ZANIERI, R. BONDI, A. BEC CIOLINI
In vitro uptake of 3H-Thymidine by tumours of the larynx

Int. J. Radiat. Biol. 51, 916, 1987

E. SCUBLA, M. BALZI, P. BOANINI, M. LADDAGA, M.G. FABRINI, A. CHIAVACCI, D. CREMONINI, A. BECCIOLINI

Parameters of cell kinetics in human oral cavity carcinomas after irradiation

Int. J. Radiat. Biol. 51, 922, 1987

A. BECCIOLINI, M. BALZI, D. CREMONINI, D. FABBRICA, V. GIACHE', S. PORCIANI

Acute intestinal radiation injury: effects of single doses and multiple fractionations

Abstr. XXXV Ann. Meet. Radiat. Res. Soc., Atlanta, USA, 77, 1987

S. PORCIANI, A. BECCIOLINI, A. LANINI, A. CHIAVACCI, E. CELLAI, S. MAGRINI

Polyamines in head and neck cancer: effects of radiation therapy

In:"Radiation Research", E.M. Fielden, J.F. Fowler, J.H. Hendry and D. Scott Eds.,
Taylor and Francis, London, vol. 1, 292, 1987
M. BALZI, A. BECCIOLINI, E. SCUBLA, A. CHIAVACCI, E. ZANIERI, E. CELLAI

The behaviour of L.I. in oral cavity carcinomas following radiotherapy

In:"Radiation Research", E.M. Fielden, J.F. Fowler, J.H. Hendry and D. Scott Eds.,
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S. PORCIANI, A. BECCIOLINI, R. BANDINELLI, A. CHIAVACCI, E. CELLAI, F. PONTICELLI, R. MUNGAI

Valori di oligoelementi in pazienti neoplastici e loro modificazioni in corso di radioterapia

Giorn. It. Chim. Clin. 12, 113-118, 1987

M. LADDAGA, M.G. FABRINI, F. CARTEI, M. BALZI, G.DI CANDIO, R. PINGITORE, A. BECCIOLINI

Modificazioni morfostrutturali da radioterpia nei tumori del retto

Atti XVII Cong. Naz. AIRB, Modena, 1987, pag. 37

A. BECCIOLINI, M. BALZI, S. PORCIANI

Il significato del dosaggio dei biomarcatori durante la radioterapia

In: Atti Simposio "I biomarcatori tumorali nella pratica clinica", a cura della XI U.S.L. GE2, 235-256, 1988

M. BALZI, O. GAZZARRINI, D. CREMONINI, A. SOMMAVILLA, E. ZANIERI, F. MELONE, A. BECCIOLINI, M.C. PAOLETTI, A. GIANNINI, C. BIAGINI
The labelling index in tumors of the human bladder

XXI Congr. Int. Soc. Urology, Buenos Aires 1988, Book of Abstract page 26

L. BANDETTINI, M. BALZI, E. SCUBLA, D. CREMONINI, A. BECCIOLINI, A. CARDINI

Cell kinetics parameters in the mucosa of patients affected by colonic tumors

XXVI World Congr. I.C.S., Milan 1988, pp. 407-410

P. BECHI, A. AMOROSI, G. CASTIGLIONE, P. ROMAGNOLI, P. MAZZANTI, A. BECCIOLINI, M. BALZI

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Directory of on-going research in cancer epidemiology, IARC Scientific Publications, Lyon, pp. 146-147, 1988

A. BENUCCI, M. TOMMASI, S. PORCIANI, M. BALZI, A. LANINI, L. BANDETTINI, A. BECCIOLINI

CEA and other tumor markers in cytosols from human colon and rectum carcinomas

J. Nucl. Med. All. Sci. 33, 70-71, 1989.

L. BANDETTINI, S. PORCIANI, A. BECCIOLINI, M. BALZI, A. LANINI, M. TOMMASI, A. CIARAMELLI, A. BENUCCI
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III Congr. Amer. Coll. Surg., Trieste, p. 79-80, OCT Publ., Trieste 1989

M. BALZI, A. BECCIOLINI, E. ZANIERI, O. GAZZARRINI, F. BOANINI, F. MELONE, A. GIANNINI, L. BRUSCHINI

Cell kinetics parameters in bladder tumors

Cell Tissue Kinet. 22, 139, 1989

P. BECHI, A. AMOROSI, R. MASPETTI, M. BALZI, E. SCUBLA, A. BECCIOLINI

The role of mucosal hyperplastic changes, cell kinetics, and bile reflux in gastric carcinogenesis after partial gastrectomy
Cancer Detect. Prev. 14, 90, 1989

A. BECCIOLINI, M. BALZI, E. SCUBLA, F. BOANINI, E. ZANIERI, P. MAURI, D. CREMONINI

Proliferative activity in human tumors

37th Ann. Meet. Radiat. Res. Soc. Seattle, Book of Abstract, p. 150, 1989

G.P. BITI, E. CELLAI, R. SANTONI, A. CHIAVACCI, S.M. MAGRINI, F. PONTICELLI, P. OLMI, A.M. PAPI

Incidence of second tumor in patients submitted to radiotherapy an analisys of 2036 cases

7th Int. Congr. Radiology, Paris, Abstract Book, Radiotherapy 2505, 1989

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-072-NL

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Title of the research contract:

Molecular-biological studies on the activation of cellular transforming genes in radiation carcinogenesis.

List of projects:

1. Role of oncogenes in malignant transformation of mouse cells.

Contract no.: B16-D-072-NL

Role of oncogenes in malignant transformation of mouse cells.

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To test the hypothesis that radiation carcinogenesis involves the separation of a proto-oncogene from a neighbouring cis-acting negative control element and subsequent translocation into the vicinity of a strong positive control element.

To characterize an oncogene rescued from a tumour cell line which has arisen after exposure of the murine fibroblast line NIH/3T3 to irradiated mouse DNA and plasmids containing the selectable marker neo^R confirming resistance to the neomycine analogue G418 as well as plasmids containing the long terminal repeat of a murine leukaemia virus. To establish whether this oncogene was also involved in the oncogenic transformation of other cell lines induced by fragmented mouse DNA.

III. Progress achieved:

MATERIALS AND METHODS

Cell culture. NIH/3T3 cells and its transfectants were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) with glutamine, supplemented with 10% heat inactivated newborn calf serum (NCS). For Re208F cells fetal calf serum (FCS) was used.

Generation of T-neo 1. T-neo 1 is a morphological transformant obtained by transfection *via* calcium phosphate precipitation of NIH/3T3 cells with a mixture of DNAs. This mixture consisted of BALB/c liver DNA which was sheared by forcing 1 ml of a 500 μg/ml solution 20 times through a 0.5 mm syringe as well as the plasmids pK0-neo and pM1sp. As judged by gel electrophoresis, the actual size of the fragments ranged from 5-25 kbp. pM1sp consists of a Mo-MLV LTR with flanking mink sequences cloned into the Eco R1 site of pBR 322. pK0-neo contains the Tn5 neomycin resistance gene in a SV40 transcription unit (courtesy of Dr. D Hanahan, Harvard University, Massachusetts), which can confer resistance to the toxic compound G418. One day after transfection cultures were split 1:9 and subjected to G418 selection (1 mg/ml). After two weeks of selection the T-neo 1 clone was picked and expanded in culture. The resulting cell line has a stable G418 resistant phenotype.

Plasmid Rescue. Two μg of restriction endonuclease digested T-neo 1 DNA was ligated (16 hours, 15 ^{0}C) using 24 units T4 ligase in 400 μl of the appropriate buffer according to instructions of the manufacturer. These conditions favor the formation of circular molecules. E. Coli strain JA221 was transformed with the ligated DNA. Ampicillin resistant colonies were selected for the criteria described in the text.

Oncogenicity assay. NIH/3T3 cells were transfected with cesium chloride purified plasmid (0.5 μg per 3.10^5 cells, seeded one day prior to transfection) and 15 μg sheared salmon sperm DNA as carrier. In the case of pT24 (positive control containing activated *ras* oncogene) 0.1 μg plasmid was used. Two weeks after transfection 10^6 cells were injected subcutaneously into a single nude mouse less than 20 weeks old. On average 5 mice were used per assay. Tumors developed within 4 weeks. Mice that did not show any tumors after 10 weeks were considered negative. Parallel cultures of transfected cells were kept for observation of morphological transformation.

Other methods. Propagation of plasmids in E. Coli strain JA221, selection using antibiotics, plasmid isolation, endonuclease treatment as well as DNA/RNA isolation and blotting experiments were all performed using standard molecular biological protocols. For RNA isolation we used the Guanidinium/Cesium Chloride protocol. Southern blots were routinely performed at 37^{0}C (50% formamide) and washed at 68^{0}C, 0.2xSSC. Northern blot hybridization was at 45^{0}C, wash at 52^{0}C, 0.15xSSC.

RESULTS

The T-neo 1 cell line. T-neo 1 is the result of transfection of NIH/3T3 cells with a mixture containing irradiated fragmented BALB/c mouse DNA, the plasmid pM1sp which harbors a Mo-MLV LTR and pK0-neo, a plasmid which can confer G 418 resistance as a selectable marker. These experiments yielded many G 418 resistant cells (3-5 clones per ng plasmid) almost all of which were not morphologically transformed. One clone gave rise to the T-neo 1 line, which has a transformed phenotype presumably caused by the action of a cellular oncogene associated with an acquired copy of the Mo-MLV LTR.

Numerous copies of integrated plasmid DNA were found in the genome of T-neo 1 cells. Southern blot hybridization with a probe that can discriminate between endogenous and exogenous Mo-MLV LTRs also revealed the presence of at least six copies of newly inserted LTR.

Plasmid Rescue. In order to isolate the sequences responsible for the oncogenic transformation, DNA isolated from this T-neo 1 cell line was digested with six different restriction enzymes and religated to form circular molecules. Transformation of bacteria yielded 50 ampicillin resistant colonies (Table I). The majority of these colonies were sensitive to

tetracycline, indicating that part of the original plasmid sequences had been lost. None of the clones was resistant to kanamycin and therefore presumably not derived from pKO-neo.

DNA was isolated from these clones and hybridized on nitrocellulose to a Mo-MLV LTR specific probe. Nineteen clones proved to contain sequences homologous to the probe. Four of these clones were analyzed in more detail. The plasmids were subjected to digestion with combinations of restriction enzymes to determine the relative position of their recognition sites. Taking the Pvu I site in the ampicillin resistance gene as a reference we were able to construct restriction maps of the plasmids. We estimate that the rescued plasmids may have lost 2.5-3 kb as compared to pM1sp, but three of them have gained 1.5-3.5 kb of unidentified DNA. We chose to examine the plasmid pSa9, which contains the largest stretch of unidentified DNA, in more detail. Its restriction map is shown in Figure 1. For comparison the map of the Mo-MLV LTR carrying plasmid pM1sp is also shown.

Occurrence and expression of the Sa9 sequence. When the Sa9 sequence originated from the transfected BALB/c DNA, one or more extra copies of the sequence would have to be present in the genome of T-neo 1. We therefore isolated the 2.5 kb Sph I fragment containing the major part of the sequence and used it as a probe in a Southern blot. NIH/3T3 contains several endogenous sequences closely related to the Sa9 sequences. The sequence has been amplified in T-neo 1.

Northern blot analysis of total cellular RNA revealed an RNA molecule of about 2 kb not present in NIH/3T3 cells which hybridized to the Sph I fragment probe. An RNA of similar size reacted with the Mo-MLV LTR probe suggesting that both sequences are present in the same transcript.

These data are consistent with the idea that Sa9 might contain (part of) an oncogene from the fragmented BALB/c DNA transcriptionally stimulated by the Mo-MLV LTR. In addition, the Sa9 Sph I fragment probe did not hybridize with the DNA of the oncogenes *abl, erb-B, fes, fms, fos, int-1, myb, myc, pim-1, H-ras, K-ras, N-ras, raf, rel, ros, sis, src* or *yes* and never found any hybridization, indicating that the putative oncogene is not homologous to any of these known genes.

Conservation of the Sa9 sequence in unrelated animal species. Extended exposure of films from Southern blots showed that sequences related to the Sa9 sequence were present in the DNA of various animal species. The sequences were most prominent in mouse and rat DNA, but were also observed in human, bovine, chicken and trout DNA, although the blot was washed at low salt (0.2xSSC). However, the required length of exposure indicates that there are few stretches of substantial homology. It appears that the putative gene from which the Sa9 sequence stems is present in quite different vertebrate species, indicating at best partial conservation.

Oncogenicity of Sa9. To test for transforming capacities we transfected the plasmid to NIH/3T3 as well as RE 208 F cells and screened for morphological transformation. Table II shows that Sa9 has at best a minimal transforming capacity. However, when cells were inoculated into nude mice two weeks after transfection, tumors appeared at the site of inoculation within four weeks. The occurrence of tumors cannot be explained by the action of the Mo-MLV LTR, since pM1sp did not have similar oncogenic potential. Involvement of the pSa9 oncogene was found at the DNA and at the RNA level. This result proves the oncogenic capacity of Sa9.

Sa9 harbors the MMTV LTR. In view of the Southern blot patterns of two mouse DNAs (BALB/c and NIH/3T3) with the pSa9 sequence as a probe compared to an MMTV LTR probe (Koornstra and Bentvelzen, unpublished results) and the occurrence of a weakly homologous sequence in other animal species, we have examined whether a relation exists between the Sa9 sequence and MMTV. Sequences related to this virus are found not only in mice, but also in humans, rats, tree shrews and several other animal species. We have compared the pSa9 sequence to the MMTV genome by hybridization with probes which represent the three most important transcription units of the virus: *gag/pol, env* and the LTR containing the 3' long open reading frame (*orf*). Spot blot analysis shows that the plasmid hybridizes to a probe from the LTR, but only slightly to a probe from the *gag/pol* region and not at all to a probe from the *env* region. In order to determine the exact location of the LTR in the plasmid we digested it with a combination of the restriction enzymes Ava I and Sph I and performed Southern blot analysis

with the MMTV LTR as a probe. There is a strong hybridization with the 1.4 kb fragment which is of sufficient size to contain an entire MMTV LTR. The 1.0 kb fragment probably contains a short stretch homologous to the MMTV LTR. Substitution of Ava I by Pst I showed the same. Figure 2 gives our interpretation of these results. The LTR is situated in the lower right quadrant. Sa9 is not unique in containing the MMTV LTR. Upon reinvestigation using spot blot hybridization with an MMTV LTR probe, 39 of the 50 rescued plasmids were found to be positive.

If the 3.5 kb acquired DNA found in the rescued plasmid stems from the sheared BALB/c mouse DNA, it must also contain MMTV LTR flanking sequences. Since neither *env* nor *gag* sequences could be detected in pSa9, we reasoned that the mouse DNA flanking the MMTV LTR must be present in at least one extra copy in T-neo 1 DNA as compared to NIH/3T3. Taq I digestion of the 2.5 kb Sph I fragment of Sa9 gives rise to two major fragments. Hybridization with an MMTV LTR specific probe shows that only the 1150 basepair fragment contains MMTV LTR sequences whereas the 850 basepair fragment does not. We have used this latter fragment as a probe on Southern blots of T-neo 1 DNA and compared it to an MMTV-LTR specific probe (table III). Several extra bands are seen in T-neo 1 DNA with the non-LTR probe indicating that the sequence is an acquired MMTV-LTR flanking sequence.

In order to assess whether the transforming activity of the Sa9 could indeed be due to the MMTV-orf sequence contained in the LTR, we have constructed several expression vectors containing MMTV-orf. These were transfected to NIH/3T3 cells. Two weeks later the cells were inoculated into BALB/c nude mice. In three separate experiments it was found that 80-100% of the inoculated mice produced fibrosarcomas within four weeks. As was the case in pSa9-induced tumours these tumours contained plasmid sequences in their cellular DNA on additional MMTV-orf sequences.

In three other tumour lines induced by irradiated fragmented BALB/c mouse DNA we observed additional MMTV-orf sequences.

DISCUSSION

We have undertaken experiments in order to gain insight into the molecular background of the transformed phenotype of cell line T-neo 1. This cell line was originated by transfection of NIH/3T3 cells with fragmented normal BALB/c DNA in conjunction with an Mo-MLV LTR and G418 resistance as a selection marker. We have used plasmid rescue as a technique to select for sequences closely associated to the Mo-MLV LTR and its flanking plasmid sequences. This approach has resulted in several clones containing various amounts of DNA not present in the original plasmids used for transfection. One of these clones, Sa9, has been studied in more detail.

The acquired sequence in Sa9 is present in T-neo 1 in several extra copies in addition to the ones endogenous to NIH/3T3. This indicates that the fragmented BALB/c DNA has served as its source. The analysis of the RNA of T-neo 1 indicates that this particular sequence is transcribed, and that the Mo-MLV LTR presumably directs this expression.

The sequence can be found in different mouse strains and various vertebrate species not closely related to the mouse, as is the case for the oncogenes studied to date.

The observed amplification and expression of the Sa9 sequence suggests its responsibility for the oncogenic phenotype of the T-neo 1 line. Amplification and overexpression have been demonstrated for several oncogenes like *myc* and *abl* . We have failed to observe substantial morphological transformation of NIH/3T3 or RE 208F cells after transfection with Sa9. Apparently this test system is not adequate, since nude mice did develop tumors after inoculation with NIH/3T3 cells two weeks after transfection with Sa9. In view of this, Sa9 must contain an oncogene. We found no homology to the oncogenes *abl, erb-B, fes, fms, fos, int-1, myb, myc, pim-1, H-ras, K-ras, N-ras, raf, rel, ros, sis, src* or *yes*, although we can not exclude the possibility that Sa9 contains a part of, or a sequence related to one of these oncogenes.

We did find hybridization to a probe from the MMTV LTR. This LTR contains a large open reading frame of which we have demonstrated the involvement in *in trans* regulation. It is easy to envisage how such a mechanism could provoke oncogenic deregulation of cellular metabolism. A similar mechanism has been proposed for the HTLV I tax gene. At present the

question remains unsolved whether the oncogenic capacities of the Sa9 sequences are brought about by expression of the LTR *trans* activator or whether alteration (perhaps mutation as is the case for the *ras* oncogene or rearrangement of the MMTV LTR sequences for instance like the ones seen in mouse T-cell leukemia underlies these phenomena.

The oncogenic capacity of exogenous MMTV-orf sequences, which are part of the MMTV-LTR, supports the view that the transactivating potential of this sequence per se contributes to the oncogenic transformation. We have recently found that the tax genes of HTLV and BLV also produce tumours in this NIH/3T3 transfection nude mouse assay.

Since we have found amplification of MMTV-orf sequence in a few other transformed NIH/3T3 lines produced after inoculation with irradiated BALB/c mouse DNA we tentatively conclude that indeed the separation of this transactivating proviral gene from inhibiting sequences under the influence of irradiation may play a role in some cases of radiation carcinogenesis.

Table I. Plasmid rescue from the T-neo 1 cell line

restriction[a] enzyme	No. Amp^r clones	No. Tet^r clones	No. LTR positive clones
Bgl II	9	0	3
Cla I	0	0	0
Hind III	4	1	1
Kpn I	3	0	1
Sac II[b]	32	2	13
Sma I	2	1	1

[a]Enzyme used to digest T-neo 1 DNA which after ligation and transformation to E. Coli was checked for resistance to ampicillin and tetracycline. The resulting plasmids were spotted on nitrocellulose, hybridized with a Mo-MLV U3 specific probe and washed at 0.2xSSC.

[b]pSa9 stems from this series.

Table II: Transforming capacity of rescued plasmid

Recipient Cell Line	Plasmid	Morphologically transformed clones (#/µg plasmid)	Tumors in nude mice three weeks after inoculation #inoculated	# with tumor
NIH/3T3	none	0.2	5	0
	pT24[a]	473	5	5
	pM1sp[b]	12	6	1
	pSa 9	19	7	6
RE208F	none	0	ND[c]	
	pT24	250	ND	
	pM1sp	8	ND	
	pSa9	10	ND	

[a]: plasmid carrying an activated *ras* oncogene.

[b]: cloned Mo-MLV LTR.

[c]: ND=Not done.

Table III: Compilation of Southern blots of T-neo 1 DNA[a] using the MMTV LTR and the non-MMTV LTR part of pSa9 as probes.

Pst I		Eco RI		Hind III	
MMTV-LTR	non-LTR[b]	MMTV-LTR	non-LTR	MMTV-LTR	non-LTR
8.8		~20		>10	
8.1		11.2		9.0	>9
6.8		8.8	9.0	8.4	
6.4	6.4	7.2	7.6	7.8	
	5.6	6.3	5.9	5.7	6.1
5.0	4.9	4.7		4.9	
4.3	4.0	4.2	4.0	4.5	4.6
3.8	3.7	3.9	3.8	4.2	4.0
	3.4	3.5	3.7	3.8	3.6
3.2	3.2	3.3	3.3	3.4	3.4
2.6	2.6	3.1	3.1	3.0	3.1
	2.3	2.8			2.9
1.8		2.3		2.6	2.7
1.2	1.2	1.9			2.5
		1.7		2.2	2.2
		1.5	1.6		2.0
		1.4			1.8
				1.1	

a: Endogenous copies as determined by Southern blot analysis of NIH/3T3 DNA are underlined.
b: 850 bp Taq I fragment of 2.5 kb Sph I pSa9 fragment.

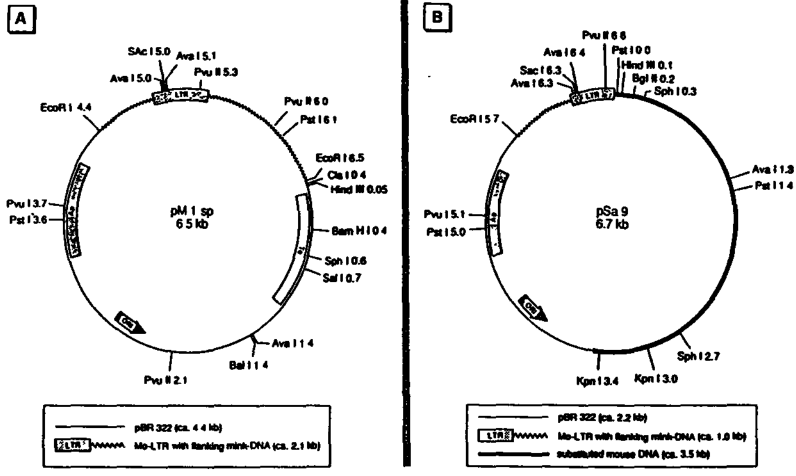

Figure 1: Restriction maps of pM1sp (cloned Mo-MLVLTR) compared to the plasmid pSa9 which was rescued from T-neo 1. Thick lines indicate unidentified DNA.

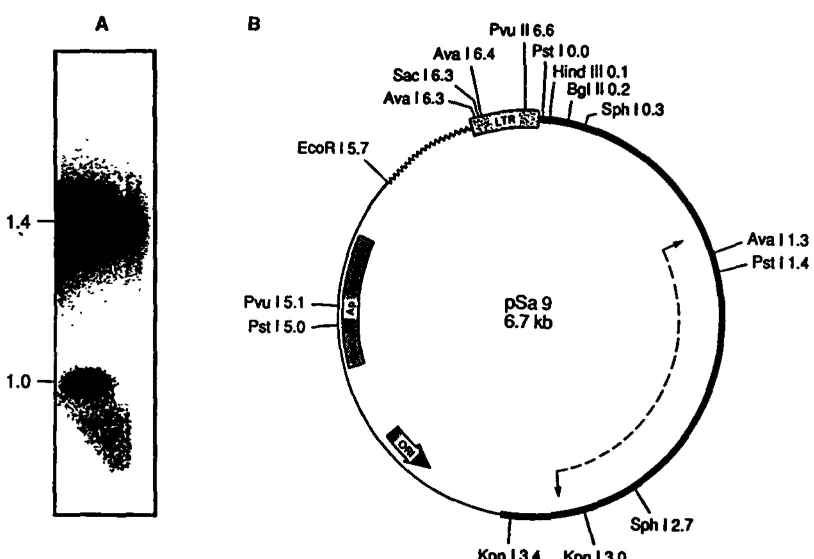

Figure 2: Presumed localization of the MMTV-orf related sequence in pSaq.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-075-NL

Radiobiological Institute TNO
Division for Health Research
Lange Kleiweg, 151
NL-2280 HV Rijswijk

Head(s) of research team(s) [name(s) and address(es)]:

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Telephone number: 015-136940

Title of the research contract:

Late effects in rhesus monkeys after whole body irradiation with X-rays and fission neutrons.

List of projects:

1. Incidence of cancer and non-stochastic diseases in an irradiated population of rhesus monkeys.

Title of the project no.:

Incidence of cancer and non-stochastic diseases in an irradiated population of rhesus monkeys.

Head(s) of project:

Prof.dr. J.J. Broerse

Scientific staff:

Prof.dr. J.J. Broerse and Dr. C. Zurcher

I. Objectives of the project:

Specific information related to the risk of radiation induced tumours or other late effects in man is limited. Data obtained from studies with larger animals and especially subhuman primates may be extremely valuable to assess the risk in man and also to estimate the RBE for tumour induction by neutron irradiation of human patients. In addition, the induction of non-stochastic effects in various tissues and the RBE of neutrons for these effects are of increasing importance for radiation protection problems. The present study on longevity, tumour induction and other late effects of total body irradiation of rhesus monkeys with fission neutrons and X rays has been in progress for about 25 years.

II. Objectives for the reporting period:

At this moment at about 4/5 of the estimated duration of the study, approximately 90 per cent of the irradiated monkeys have died compared with 50 per cent of the control group. All remaining monkeys receive a physical examination each month by a veterinarian with extensive experience with non-human primates for the clinical presence of tumours, cataract formation and changes in general condition.
The necropsy results over the total contract period are reported and the diagrams for malignant tumour development are updated. Risk factors and RBE values are derived from the tumour incidence results.

III. Progress achieved:

Introduction

The two groups of long-term surviving irradiated monkeys are part of a study on the effectiveness of bone marrow transplantation to prevent death due to the haemopoietic syndrome (Broerse et al., 1978). One group of long-term survivors consisted of nine macaques irradiated with fission neutrons with doses ranging from 2.3 to 4.4 Gy (average dose 3.4 Gy) and the other of 20 X-irradiated macaques which received doses between 3.0 and 8.6 Gy (average dose 6.7 Gy). A third group of 21 untreated rhesus monkeys of comparable age distribution was maintained under identical husbandry conditions to serve as a control group.
The tumour incidence and post-irradiation observation periods for rhesus monkeys are shown in Figures 1 and 2. Time of irradiation and time of occurrence of tumours are indicated. Lines ending in cross bars signify death and arrow heads denote that the monkeys are still alive.

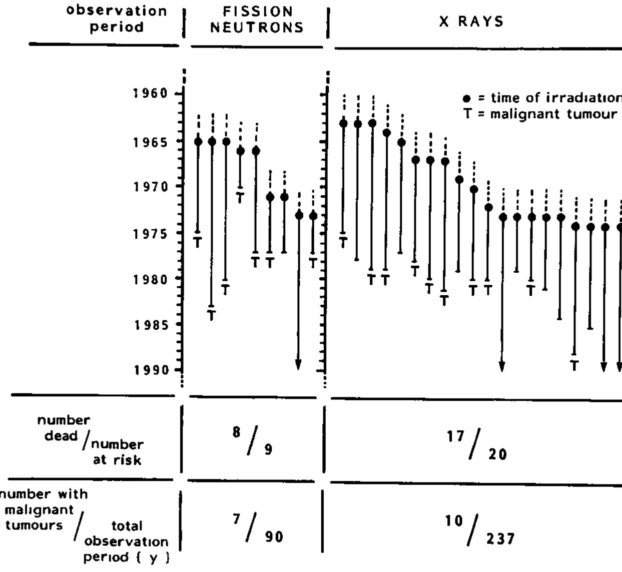

Figure 1: Tumour incidence in long-term surviving rhesus monkeys after irradiation and bone marrow transplantation.

Necropsy results

Rhesus monkey no. 730, an untreated control, born in 1961, died after a two-month period of increasing anorexia and weight loss on December 21, 1984. A necropsy and complete histological examination was performed. The clinical findings and the emaciation at death could be explained by an extensive saucerlike ulcerative carcinomatous process (diameter 7 cm) in the cardia of the stomach, extending into the lower esophagus, pancreas and spleen. A large metastasis (diameter 6 cm) with central necrosis was found in the liver. Other tissues did not show significant changes and no other metastases were observed.

In the monkey no. 646, irradiated with 2.6 Gy fission neutrons in 1965, a glomus tumour of the right scrotum and 2 subcutaneous nodules, one neurofibroma and the other neurofibromatosis, were removed in 1979 and 1982, respectively. The general condition of this animal

gradually deteriorated after Mantoux testing in November 1984.
At that time, in association with stretching of the hind legs during anesthesia, subcutaneous hemorrhages occurred in both inguinal regions. Thereafter, the hemorrhagic area got infected with focal ulceration. The local inflammatory process appeared to be resistant to any therapy and the animal died, presumably due to sepsis, on January 24, 1984. The gross necropsy revealed large hemorrhagic areas in both inguinal regions with discoloration and softening of the underlying adductor muscles and focal ulceration of the covering skin, status after extirpation of the right testicle and scrotal glomus tumour and a number of other lesions in lungs, kidneys, liver, adrenal gland, thyroid, pituitary and ischiadic nerve. In addition, many tissues (muscle of left thigh, skin of left foot, meninges, thyroid, mucosa of intestinal tract) showed small, well circumscribed hemorrhagic areas ranging from 1.0-0.3 cm diameter. Between the first and second finger of the left foot an hemorrhagic area with ulceration of the overlying skin was observed.

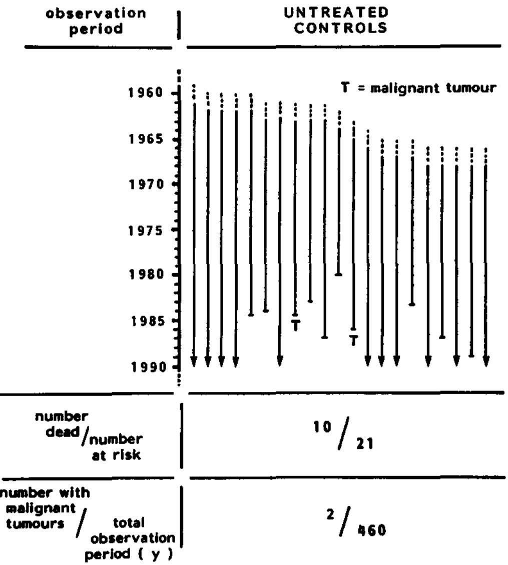

Figure 2: Long-term surviving monkeys in the control group.

After microscopic examination, the following diagnosis could be made: renal cortical carcinoma, diameter 1.3 cm, right kidney; pheochromocytoma 0.5 cm, right adrenal; pancreas, multiple small insulinomas; schwannoma of left ischiadic nerve; thyroid: cystic follicles, interstitial fibrosis and multifocal folicular hyperplasia; pituitary: multifocal hyperplasia in distal part. No glomus tumour metastases were observed.
In addition to these proliferative lesions and of more relevance to the clinical condition were the histological signs of a long-standing process of increased intravascular coagulation with sometimes extreme narrowing of the lumen of small and large arteries due to myointimal fibrosis. Ischemic necrosis and fibrosis secondary to impaired vascular sypply were found in the kidneys, lungs and liver.
It can not be excluded that this vascular disease process already existed for many years as severe myointimal fibrosis and organizing thrombi were observed already in 1979 in the

peritesticular tissue in the vicinity of the glomus tumour and in the cavernous hemangioma of the left thigh. The grossly observed hemorrhagic areas in various tissues at necropsy appeared to be teleangiectatic veins and capillaries and multifocal cavernous hemangiomas. The rupture of such a hemangioma could explain the large hemorrhage in the inguinal region after stretching of the hind legs during anesthesia in November 1984 (which event started the clinical deterioration). Bacteriological examination of heartblood and inguinal ulcer revealed the presence of Staphylococcus aureus.
It can be concluded that the rhesus monkey no. 646 died from diffuse intravascular coagulation and terminal septicemia with extensive endarterial myofibroblastic proliferation resulting in severe ischemic necrosis and fibrosis in kidneys, liver and lung. Other findings not directly related to the death of the animal and probably caused by the irradiation were: renal adenocarcinoma, adrenal pheochromocytoma, multiple panchreatic insulinomas, multiple hemangiomas and perineural sheath tumours.

The rhesus monkeys no. 2658, irradiated with 8.5 Gy X rays in 1973, suffered from intractable diarrhea during the last month of its life. Fecal examinations for protozoa or bacteria remained negative. During the last week serum uerum and creatinin values increased. The animal died on March 6, 1985. Necropsy revealed a stricture in the distal colon and several adhesions between intestinal loops in that area. The colon proximal of the stricture was severely dilated and the lumen was filled with fluid and gas. At microscopical examination, adhesions and a stricture of the distal colon appeared to be caused by endometriosis of the colonic serosal surface and associated inflammation and fibrosis. No other significant lesions were observed. Bacteriological examination of heartblood revealed the presence of Staphylococcus aureus.

The non-irradiated female monkey, no. 926, born in 1963, developed a diarrhea with severe mucus production in the middle of December 1985. Examination of the faeces revealed the presence of flagellates. Treatment with Flagyl had no effect. The general condition graduallly deteriorated, blood urea and serum creatinin levels increased and she was euthanized on January 23, 1986. At gross morphology, the tissues were dry. No significant lesions were observed except a multifocal yellow discoloration of the liver.
Microscopic examination revealed the presence of a mild chronic colitis with goblet cell hyperplasia, a moderate chronic gastritis, mild to moderate fatty changes in the liver, mild multifocal renal tubular atrophy and mild interstitial renal cortical fibrosis. The larger renal arteries shown the presence of a mild to moderate intimal fibrosis. As an incidental finding, a small anaplastic carcinoma of the uterine cervix was observed. Metastases have not been detected. This animal died of intractable diarrhea due to chronic colitis and subsequent fluid loss.

The non-irradiated male monkey, no. 1747, born in 1966, died in March 1987 of acute right cardiac failure. At necropsy and histology, an extreme hyperplasia of the muscular wall of the large pulmonary arteries was observed compatible with a diagnosis of pulmonary hypertension. The right cardiac centricle was dilated and the liver showed signs of acute congestion. Tumours were not observed.

The non-irradiated male monkey (Balthazar), born in 1961, died in February 1987, due to respiratory failure. This monkey had an excessive thoracic kyphosis at Th 5-6 for years. At necropsy, severe lung lesions were present. There was extensive lung mite infection with bronchiectasis, lung fibrosis and emphysema. The large systemic arteries showed focal intimal fibrosis and a variable narrowing of the lumen. Left and right cardiac ventricles were hypertrophic. Other lesions observed were multifocal, fibrotic thickening of the liver capsule with periportal and perisinusoidal fibrosis in the subcapsular area and chronic gastritis. Tumours were not observed.

The X-irradiated female monkey, no. 2490, born in 1971, was euthanized in April 1987, because of technically inoperable uterus myomatosus. Necropsy and histological examination revealed, in addition to the presence of multiple benign leiomyomas of uterine body and cervix, also a cavernous hemangioma of the left adrenal, a benign splenic stromal tumour, a severe

chronic gastritis and fibrosis and mineralization of the pancreatic islets.

The X-irradiated female monkey, no. 2489, born in 1971, was euthanized in October 1987 because of increasing weight loss and intractable diabetes. Necropsy and histological examinations revealed fibrosis and mineralization of pancreatic islets but no other histological lesions compatible with longstanding diabetes were observed. The liver showed multifocal areas of fatty change. In the lungs, focal mild interstitial fibrosis was found. No neoplastic lesions were observed.

The male rhesus monkey, no. 2652, , TBI X-irradiated in January 1974, died unexpectedly at 24 July 1988 after a few days of clinical illness at the age of 17 years. His body weight had decreased during the last year from 4500 to 3500 g. At necropsy and histological examination it appeared that he died from severe acute bronchopneumonia, secondary to severe pneumonyssus infection of the bronchial tree with fibrosis, bronchiectasis and emphysema. Septic abcesses with cocci were observed in the liver. In the right and left kidney papillary cortical carcinomas were observed (with diameters of 1.5 cm and 0.4 cm). Additional non-neoplastic findings were: right sided cataract, severe testicular atrophy, mild glossitis and oesophagitis due to candida infection; severe nodular hyperplasia and fibrosis in the right lobe of the liver; localized venectasies in the meninges with recent and organised thrombi, moderate to severe renal interstitial fibrosis and multiple smaller and larger cysts at the cortico medullary junction.

The female rhesus monkey, no. 2498, treated with TBI X rays in November 1972, was euthanized December 12, 1988 at the age of 18 years, because of extreme emaciation (2100 g) and decline of general condition. At gross morphology only minor lesions, apart from the general wasting, were observed. Microscopically a small papillary cortical carcinoma was observed in the left kidney and a tubular adenoma in the right kidney. Uterine abnormalities were: multiple small leiomyomas, an endometrial polyp and multiple foci of endometriosis at the serosa. The most important non-neoplastic lesions were: severe septal fibrosis of the heart, severe fibrosis and atrophy of the exocrine pancreas with vacuolar degeneration of islet cells and moderately severe chronic superficial gastritis. These lesions could explain the clinically severe wasting disease. Additional non-neoplastic findings were: chronic liver-congestion and liver cell atrophy, glossitis and oesophagitis due to candida, severe lymphoid tissue atrophy, renal cortical cysts and moderate interstitial fibrosis.

During the last year only one of the control rhesus monkeys, no. 1752, died. This animal was clinically healthy and died accidentally due to asphyxia after ketamine anesthesia necessary for scheduled Mantoux testing and blood sampling. Histological examination revealed diffuse myocardial fibrosis and myocardial cell hypertrophy and moderate multifocal emphysema and diffuse interstitial fibrosis of the lungs. In addition, there were histological signs of mild chronic colitis and superficial gastritis.

Conclusions

In the groups of rhesus monkeys irradiated with high doses of X rays (average dose 6.7 Gy) and fission neutrons (average dose 3.4 Gy) an appreciable number of malignancies have been observed. In a number of cases the animals died with multiple tumours of mutually different types. The tumours observed include glomus tumours, osteosarcomas, renal adenocarcinomas, thyroid follicular carcinomas and central nervous system tumours (astrocytoma and glioblastoma). In the group of 21 control monkeys, two monkeys died with malignant tumours. The latency periods for induction of neoplastic diseases varied between 7.5 and 16 years for X-irradiated animals and between 4 and 18 years after neutron irradiation. As a consequence of this study, it is recognized that there is a strong need to screen regularly for secondary tumours, which may arise in patients who have previously received high-dose whole-body irradiation.

The results can be analysed in terms of the number of animals developing tumours per group as a function of the total observation period for the entire group. In the total observation period of 90 monkey-years, seven neutron-irradiated monkeys developed malignant tumours.

For the X-irradiated monkeys, ten have died with malignant neoplasms during the observation period of 237 monkey-years, while two animals died with malignant neoplasms in the control group during 460 monkey-years. The simplest way to obtain a quantitative assessment of the risk for tumour induction is to divide the number of observed tumours by the years at risk and the average absorbed dose. In this way, risk factors of $10 \times 237^{-1} \times 6.7^{-1} = 63 \times 10^{-4}$ Gy^{-1} $year^{-1}$ for X rays and $7 \times 90^{-1} \times 3.4^{-1} = 229 \times 10^{-4}$ Gy^{-1} $year^{-1}$ for fission neutrons and an RBE of approximately 4 can be derived. It should be realized that these risk factors are derived from tumour incidence data obtained at relatively high doses and that they pertain to risk factors per monkey-year. Furthermore, this appraoch does not take into account the time-dependence of the tumour appearance. If one applies the concept of cumulative tumour rate the cumulative hazards are 0.64 and 0.18 for the neutron and X-ray experiment at 10 years past irradiation. On the basis of such a calculation the RBE for tumour induction in rhesus monkeys would be equal to $0.64 \times 3.4^{-1}/(0.18 \times 6.7^{-1}) = 7$. It is clear that the different approaches can produce some ambiguities in the assessment of risks resulting from different types of radiation.
For a comparison of the risk factors for induction of fatal malignancies in subhuman primates, it should be realized that the life span of monkeys is approximately one third of that in man. On the basis of recent epidemiological studies risk coefficients have been derived for occurrence of fatal malignancies after exposure of man to high doses of low-LET radiation (Broerse and Dennis, 1990).With the additive risk projection model the lifetime risks of mortality vary between 4 and 5×10^{-2} Gy^{-1}, whereas the multiplicative model results in a lifetime risk between 7 and 11×10^{-2} Gy^{-1} for the whole population. These risk estimates apply to a dose range of 0.5-6 Gy and they are strongly influenced by the finding that children are considerably more sensitive to radiation effects than are adults. Assuming a mean latency period of 20 years for the manifestation of malignancies the yearly risks would vary between 20 and 55×10^{-4} $year^{-1}$. It is interesting that the risk factor of 63×10^{-4} Gy^{-1} derived from the monkey experiments is resembling the human experience.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

This study is jointly performed by the Radiobiological Institute TNO and the Institute for Experimental Gerontology TNO.For the dosimetric aspects of the irradiations and the pathology, European collaboration has been established within the framework of the European Late Effect Project Group (EULEP committees for dosimetry and pathology).

V. Publications:

- Broerse, J.J., Van Bekkum, D.W., Hollander, C.F. and Davids, J.A.G. Mortality of monkeys after exposure to fission neutrons and the effect of autologous bone marrow transplantation. Int. J. Radiat. Biol. **34**, 253-264, 1978.
- Broerse, J.J., Hollander, C.F. and Van Zwieten, M.J. Tumour induction in rhesus monkeys after total body irradiation with X-rays and fission neutrons. Int. J. Radiat. Biol. **40**, 671-676, 1981.
- Broerse, J.J., Hennen, L.A. and Van Zwieten, M.J. Radiation carcinogenesis in experimental animals and its implications for radiation protection. Int. J. Radiat. Biol. **48**, 167-187, 1985.
- Broerse, J.J., Van Zwieten, M.J. and Zurcher, C. Carcinogenic risk of the medical use of X rays. Proc. 6th Annual Meeting of the European Society for Therapeutic Radiology and Oncology (ESTRO), 222, Lisboa, 1987.
- Broerse, J.J. Tumour induction in monkeys and rats after X- and neutron-irradiation. In Proc. Int. Conf. on Biological Effects of Large Dose Ionizing Radiation. Chinese Medical Association 319-337, 1988.
- Broerse, J.J., Van Bekkum, D.W. and Zurcher, C. Radiation carcinogenesis in experimental animals. Experientia, **45**, 60-68, 1989.
- Broerse, J.J. and Dennis, J.A. Dosimetric aspects of exposure of the population to ionizing radiation. Int. J. Radiat. Biol. 1990. (in press).

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-219-NL

Academisch Ziekenhuis Leiden
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Head(s) of research team(s) [name(s) and address(es)]:

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Telephone number: 071-261990

Title of the research contract:

Analysis of dose-effect relations for radiation carcinogenesis by various mathematical models.

List of projects:

1. Mathematical-statistical evaluation of data from animal and human studies and further development of statistical methods.

Title of the project no.:

Analysis of dose-effect relations for radiation carcinogenesis by various mathematical models.

Head(s) of project:

Prof.dr. J.J. Broerse

Scientific staff:

Prof.dr. J.J. Broerse, Dr.ir. J. Davelaar and Ing. J. Weeda.

I. Objectives of the project:

Quantitative estimates of the risk for breast cancer induction are essential for risk-benefit-cost analysis of mammography procedures. Large scale programmes on radiation-induced mammary neoplasms have been performed at relatively few laboratories in Europe and the United States. The results obtained at these institutes are analyzed by mutually different statistical models and associated computer programmes. A collaboration has been established between the institutes in the Netherlands and in the Federal Republic of Germany, to investigate the implications of the different approaches. Special emphasis will be placed on the extrapolation of the animal data, to radiogenic risks of ionizing radiation for the human situation.

II. Objectives for the reporting period:

Experimental results on induction of mammary tumours in the rat for different exposure conditions (single and fractionated irradiation, sometimes combined with hormone administration) were available from earlier experiments. Different methods and models were applied on the experimental data from these large scale animal programmes with the aim of assessing the dependence on dose and exposure conditions with radiation carcinogenesis as the biological endpoint. The statistical methods were incorporated in an analysis program developed for a personal computer.

III. Progress achieved:

RISK ASSESSMENT OF RADIATION CARCINOGENESIS IN EXPERIMENTAL ANIMALS

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Abstract

Appropiate statistical analyses have been applied to estimate the carcinogenic effect of radiation on rats. These methods consisted of a maximum likelihood or fit procedure to an analytical Weibull function as well as the use of the proportional hazards model. A comparison between the methods show the results to be independent of the applied analysis technique within the errors. The derived methods are used to assess carcinogenesic risks in animal experiments as a function of dose, radiation schedule, such as single dose and fractionated irradiation, and radiation quality. An RBE of around 0.5 is found for carcinogenesis due to Cs-137 gamma rays as compared to 300 kV X-rays.

Introduction

The carcinogenic effect of ionizing radiation for low dose levels has been investigated in a number of studies in the laboratory, mainly due to the lack of human epidemiological data. In these animal cohorts endpoints are taken as the occurrence of a malignant (or benign) tumor or a general lifetime shortening. Different analysis techniques were proposed for these carcinogenesis data with the aim to evaluate the dependence on dose, radiation schedule and radiation type (Kellerer and Chmelevsky, 1982; Broerse et al 1985). A systematic comparison of these various analysis techniques on the same set of animal data has up to now not been undertaken. However it is important to elucidate the above mentioned dependences, particularly in data sets with limited statistics. Different analysis techniques have been applied to animal data from the Radiobiological Institute TNO, which were performed with the specific aim to investigate the risk of mammography procedures. These analyses will test previous findings in an attempt to compare the results from the various models and will provide new information on the RBE for carcinogenesis of gamma rays, which will also be discussed in the context of radiation protection applications.

Methods

The type of animal data for radiation carcinogenesis discussed in this paper, consists of the observation of a tumor (benign or malignant) at a certain time. The distribution of these time periods up to the occurrence of a tumor, also denoted as 'failure' times, can be expressed as a survivor function S(t) or the cumulative hazard function H(t) (Kalbfleisch and Prentice 1980). These two functions are related as follows:

$H(t) = -\log(S(t))$.

S(t) will be influenced by concurrent deaths of animals through causes not related to the endpoint, i.e. without the occurrence of a tumor. This right-censoring can be corrected for by actuarial analysis by the sum or product limit estimate (Kaplan and Meier 1958). An experimental assumption is made for microtumours observed upon obduction, which are taken to represent failures at a time of 10 weeks thereafter (Broerse et al., 1986).

The resultant survivor function can be analyzed by parametric models such as the Weibull distibution. Alternative distributions have also been proposed: exponential, log-normal, gamma or log-logistic distributions (Kalbfleisch and Prentice 1980). The survivor function, represented as a Weibull distibution, has the following form:

$S(t) = \exp[-\{(t-g)/\alpha(D)\}^{\beta}]$,

where g is the time offset, α is the time scale parameter as a function of dose D and β is the shape parameter. The optimization of this Weibull function to the survivor data can be achieved through a x^2 fit (Broerse et al. 1985) or the maximum likelihood method (Kellerer and Chemelevsky 1982). The shape parameter β is assumed to be the same for the common fit to the survivor functions under study. The parameter g is taken to be zero for all analyses. A good assessment of the validity of the assumption, that the survival curve is represented by a Weibull function, is a log-log plot of the cumulative hazard, which should follow a straight line. The relative hazard of two cohorts is defined as:

$h(D)=\{\alpha(0)/\alpha(D)\}^{\beta}$.

The survivor functions have also been analyzed by non-parametric models: (i) the log-rank test (Mantel 1966), giving a measure through the p-value of the equality of two (or more) survivor functions and (ii) the proportional hazards model (Cox 1972), giving a direct quantitative measure of the relative hazard h(D) in the animal cohorts under study without any assumption on the actual shape of the survivor function.

Results

For an assessment of the relative hazard a comparison needs to be made with a control cohort. Differences exist for control groups over the years, as shown in figure 1a for the WAG/Rij animal control cohorts from the TNO experiments since 1974 up to 1982. The vertical separation would represent a presumed difference in the relative hazard between the cohorts. A log-rank test shows a statistical difference between the cohorts in 1974 and 1978 ($p=0.007$), however no statistical difference exists for the other years. As a consequence of the variation over the years of experiment the analysis of the irradiated animals needs to taken against the control of the same year. The analysis with a common Weibull function to the control groups is shown in figure 1. The Weibull function as a straight line is a reasonably good representation of the cumulative hazard data.

The above outlined analysis techniques are applied to animal cohorts from the experiments at TNO in the year 1982. These experiments were meant to investigate the carcinogenic action in the mammary gland of female WAG/Rij rats. A number of experimental factors were varied for the cohorts in these studies: (i) relatively low dose levels were employed notably 0.3 Gy and 1.2 Gy, both as a single dose as well as fractionated over 120 fractions, which would simulate the procedure in mammographic screening; (ii) the single dose radiation was applied at two distinct times in the lifespan of the rats, i.e. at an age of 8 and 17 weeks to investigate a possible age dependence; (iii) the application of a natural hormone E-2 to simulate the carcinogenic risk of combined anticonceptiva and radiation. Further experiments were performed with the benign fibroadenomas, which will however not be discussed here (Broerse et al. 1989). The relative hazard, as defined above, for carcinomas versus the control group is shown in figure 2 for the various animal cohorts from the 1982 experiment with WAG/Rij rats, subjected to total body irradiation of gamma rays. The data are analyzed by three different methods, i.e. the chi-square fit, the maximum likelihood optimization and the proportional hazards model. From this figure it can be concluded, that within the errors the relative hazard is fully consistent for the three analysis methods and that the errors seem to vary slightly with respect to the applied method, being somewhat smaller in the case of the Weibull fitting procedure.

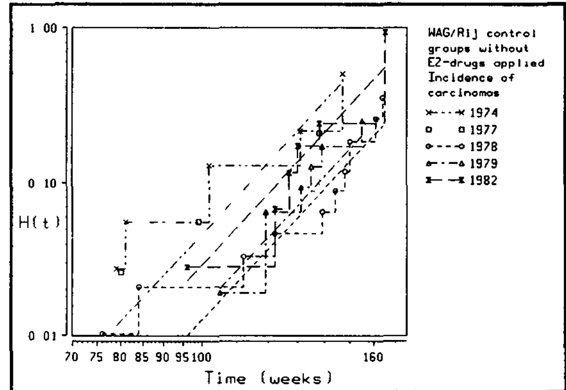

Figure 1: Control group versus year of experiment.

The relative hazard for carcinogenesis can be seen to increase with dose from figure 2. The largest hazard is obtained at 1.2 Gy for the animals irradiated at an age of 8 weeks. The fractionated data show almost no difference from the control group with the relative hazard around 1 for both the 0.3 Gy and 1.2 Gy irradiated cohorts. These findings are consistent for all three analysis methods. An extensive discussion of the dose-response relationships in terms of linear and linear-quadratic models can be found in Broerse et al. (1989), where the data were analyzed with the chi-square fitting procedure.

The relative hazard for the same experimental conditions with the additional administration of the E-2 hormone is shown in figure 3. Again the three analysis methods provide consistent values for the relative hazards within the errors with the smallest error bars for the chi-square fitting procedure. A marked increase in the relative hazard is found for the cohort of animals irradiated at 8 weeks of age, indicating a potentiating action of the E-2 hormone in combination with irradiation. The dose-effect relation can only be described in this case with a quadratic model (Broerse et al. 1989). For the cohort irradiated at 17 weeks of age the relative hazard is also increased with respect to the situation without administration of the E-2 hormone. The fractionated data still show almost

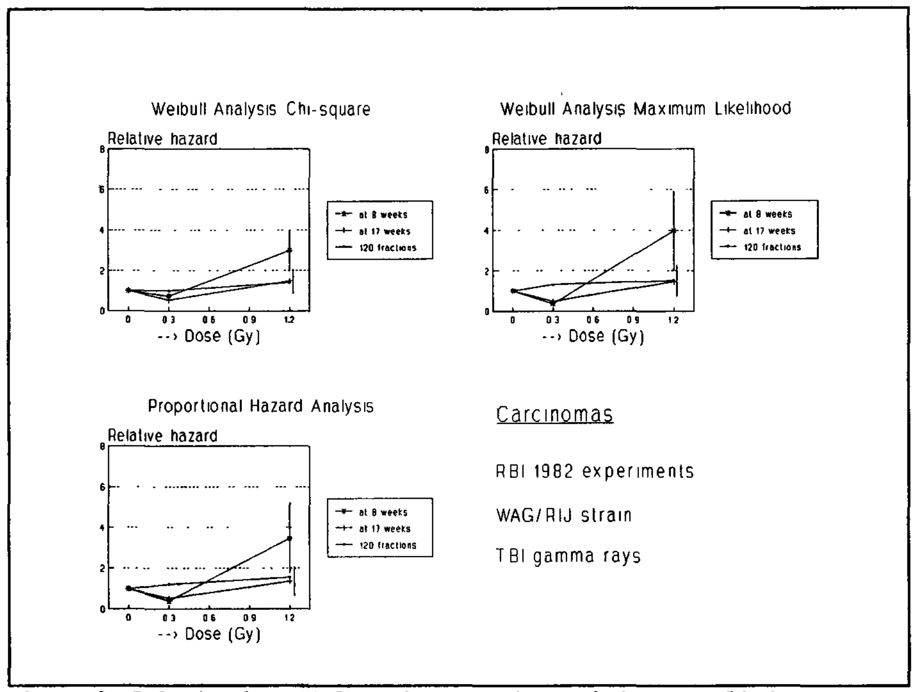

Figure 2: Relative hazard for cohorts without E2-drugs applied.

no increased hazard as compared to the control group.

The dependence of the carcinogenic risk on radiation quality has been investigated from data of total body irradiation by gamma-rays, as in the previously described experiments, in comparison with 300 kV X-rays. For this calculation of the relative biological effectiveness (RBE) both the malignant carcinomas and benign fibroadenomas have been used. The RBE as a function of dose is shown in figure 4 for similar cohorts as above irradiated at 8 and 17 weeks of age with a single dose, in addition to the fractionated experiments. As can be seen from this figure the RBE of gamma rays shows a relatively large value for the carcinomas in the cohort irradiated at 8 weeks, but consistently lower values for the other data, which in fact are compatible with a value around 0.5.

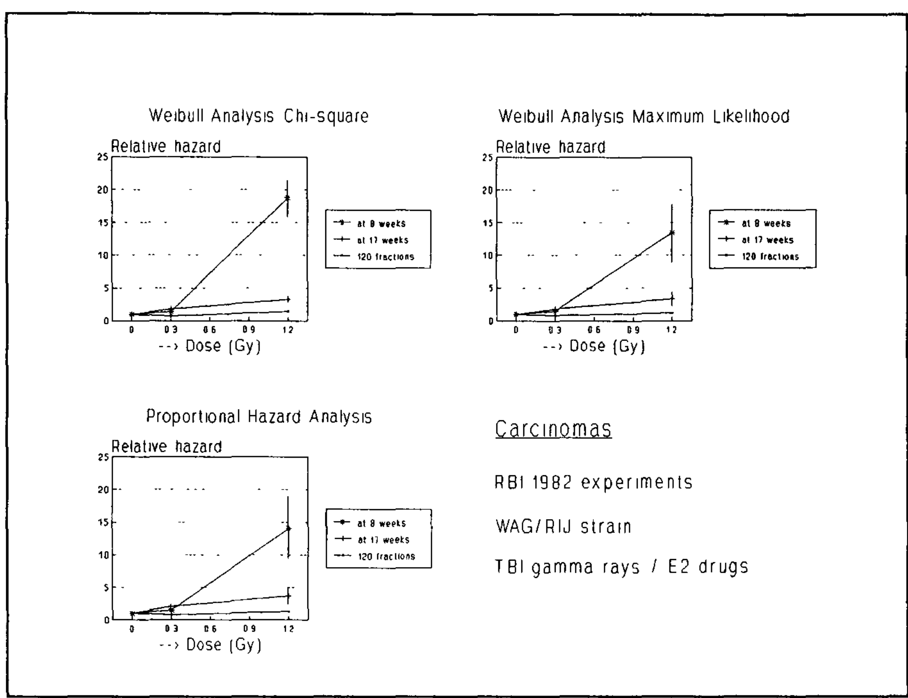

Figure 3: Relative hazard for cohorts with E2-drugs applied.

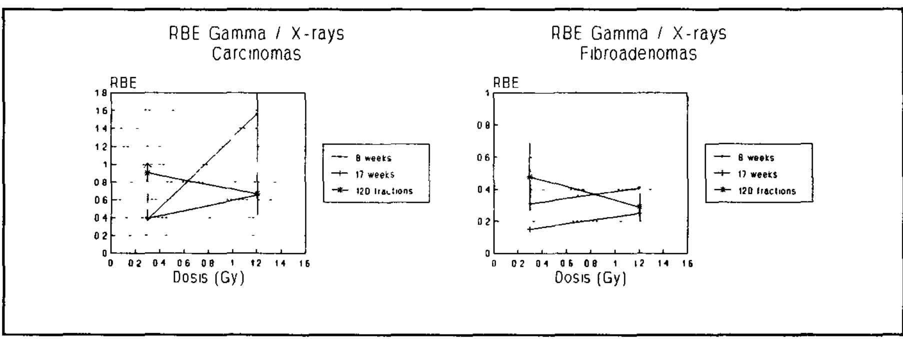

Figure 4: Relative biological effectiveness for gamma-rays.

Conclusion

The analysis methods employed show consistent results for the described experiments, but this may not be warranted for all types of carcinogenic experimental conditions. Such an implication probably means that additional tests will have to be performed on other data sets.

For the specific endpoint notably mammary carcinogenesis in the rat the following conclusions can be formulated: (i) the dose-effect curves for induction of carcinoma can be described by a quadratic relationship, (ii) a clear age dependence has been demonstrated with a larger susceptibility for mammary carcinogenesis in younger animals, (iii) the tumour incidence data are appreciably lower after the fractionated irradiations than for single irradiations at the same dose level. These findings imply that risk factors for induction of mammary carcinomas obtained at relatively high doses, can be reduced for the dose levels encountered in screening procedures such as mammography.

The relative biological effectiveness (RBE) of gamma-rays with reference to X-rays varies between 0.8 and 0.9 for acute somatic effects such as cell survival and the occurrence of radiation syndromes in experimental animals. The quality factor (Q) introduced for low dose levels and mainly valid for the causation of stochastic effects, is also expressed with reference to X-rays. On the basis of the reported studies an RBE of 0.5 was derived for induction of mammary carcinoma after gamma irradiation. When the quality factor concept would be related to gamma irradiation at fractionated exposures, an appreciable raise in Q values could be anticipated.

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IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

- Institut für Medizinische Strahlenkunde, University of Würzburg, Federal Republic of Germany (A.M. Kellerer).
- Institut für Strahlenschutz GSF, Neuherberg, Federal Republic of Germany (D. Chmelevsky and H.G. Paretzke).
- Radiobiological Institute TNO and Institute for Experimental Gerontology TNO (D.W. van Bekkum, J. Zoetelief and C. Zurcher).

V. Publications:

Broerse, J.J., Hennen, L.A. and Van Zwieten, M.J.: Radiation carcinogenesis in experimental animals and its implications for radiation protection, Int. J. Radiat. Biol. 48, 167, 1985.

Broerse, J.J., Hennen, L.A. and Solleveld, H.A.: Actuarial analysis of the hazard for mammary carcinogenesis in different rat strains after X- and neutron-irradiation, Leukemia Research, 10, 749, 1986.

Kellerer, A.M. and Chmelevsky, D.: Analysis of tumour rates and incidences, a survey of concepts and methods. In: Neutron Carcinogenesis, EUR 8084 (J.J. Broerse and G.B. Gerber, eds.), 209, 1982.

Broerse, J.J.: Influence of physical factors on radiation carcinogenesis in experimental animals. In: Low Dose Radiation (K.F. Baverstock and J.W. Stather, eds.), 181, 1989.

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Davelaar, J., Broerse, J.J., Weeda, J., Chmelevsky, D.: Analysis of dose-effect relations for carcinogenesis in experimental animals, 22nd Annual Meeting, European Society for Radiation Biology, Brussels, 1989.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: B16-D-088-F

Commissariat à l'Energie
Atomique, CEA
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Telephone number: 75-50.43.80

Title of the research contract:

Studies into the actual toxicity of uranium compounds under conditions prevailing in the industry with a view to re-examining ICRP norms.

List of projects:

1. Studies into the actual toxicity of uranium compounds under conditions prevailing in the industry with a view to re-examining ICRP norms.

Title of the project no.:

Etude du métabolisme de la toxicité réelle des composés d'uranium en situations industrielles en vue du réexamen des normes de la CIPR.

Head(s) of project:

Dr. J. Chalabreysse

Scientific staff:

E. Ansoborlo - M. Archimbaud - P. Bérard - M.H. Hengé-Napoli - E. Pujol

I. Objectives of the project:

Evaluer les risques réels en déterminant les caractéristiques physico-chimiques et les comportements métaboliques des composés d'uranium produits ou traités dans l'industrie nucléaire : Développement d'une méthodologie.

II. Objectives for the reporting period:

Cette méthodologie est la suivante :

- Caractérisation physico-chimique d'un certain nombre de composés industriels de l'uranium utilisés comme UF_4, UO_2, diuranates calcinés et UO_3.
- Etude et développement d'un test de solubilité in vitro chimique avec utilisation de différents solvants contribuant à la solubilisation des poussières.
- Développement d'un test de solubilité in vitro cellulaire sur macrophages alvéolaires de rats.
- Intoxication par voie intratrachéale de rats avec des composés industriels de l'uranium.
- Etudes in vivo par inhalation sur rats et suivi des excrétions urinaires ainsi que sacrifice et analyse des organes et tissus en fin d'expérimentation et tentative de bilan d'adsorption dans les organes et tissus.
- Chez l'homme, essai de corrélation entre les études d'atmosphère de postes de travail et les résultats obtenus en radiotoxicologie.

III. Progress achieved:

III.1 - Etude des caractéristiques physico-chimiques

L'expérience acquise pendant plusieurs années sur les composés de l'uranium a permis de développer l'utilisation de différentes méthodes permettant l'étude des principales caractéristiques physico-chimiques.

Methodology

Les techniques de caractérisation principalement utilisées sont : les rayons X pour la forme cristalline, l'infra-rouge solide pour le type de liaison (uranyle par exemple), l'ESCAE pour déterminer les niveaux d'énergie des liaisons atomiques, la Microscopie Electronique à Balayage (MEB) pour visualiser les particules et déterminer leur forme, la fluorimétrie pour doser l'uranium présent dans un composé et enfin la spectrométrie de masse pour déterminer la composition isotopique.

Results

Cette méthodologie a été appliquée à chacun des composés suivants étudiés pendant ces 5 années, soit : UF_4, UO_2, uranates calcinés (QML et NUF) et UO_3.

- pour UO_2 la mise en évidence au MEB de particules intrinsèques très fines (< 1 µm) mais sous forme d'aggregats.

- pour les uranates calcinés QML et NUFCOR l'analyse RX a permis de différencier ces 2 composés l'un étant plutôt de l'U_3O_8 (QML) et l'autre U_3O_8 + UO_x (NUFCOR).

- enfin, pour UO_3 industriel nous avons mis en évidence d'une part 3 composés présents (ADU (<5 %), U_3O_8 (<5 %) et principalement UO_3) par RX et l'UO_3 séparé a été caractérisé en IR. Cette approche permet de mettre en évidence un vieillissement dans le temps dû à un phénomène d'hydratation de la poussière.

Discussion

Ces différentes techniques et ces quelques résultats marquants ont mis en évidence l'importance de la caractérisation préalable aux études in vitro et in vivo. Nous verrons que le problème d'hydratation de l'UO_3 par exemple influe beaucoup sur la transférabilité du composé.

III.2 - Etude de solubilité in vitro chimique

Cette étude s'est déroulée en plusieurs étapes qui ont permis d'une part de comparer différents types de tests (dynamiques et statiques) et d'autre part divers solvants, ceci afin de se rapprocher le plus possible des tests in vitro cellulaires et des phénomènes observés in vivo (animal, homme).

Methodology

Le test retenu pour ce type d'étude a été le test statique plus simple d'utilisation (fig. 1).

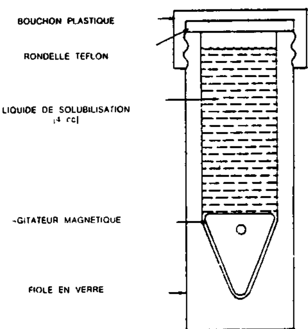


Figure 1 : Système de dissolution in vitro statique

Les solvants les plus intéressants qui ont été étudiés sont :

- solution de bicarbonates,
- solution de phosphates,
- solution de Gamble avec barbotage d'oxygène,
- solution de Gamble avec addition de pyrogallol (p) et de Super-Oxyde Dismutase (SOD) sans barbotage d'oxygène (formation d'ions superoxyde O_2^-),
- Milieu de culture cellulaire (BME) sous 5 % de CO_2.

Ces tests sont réalisés en température à 37°C. Les cinétiques observées sont exprimées sous forme d'exponentielles permettant de calculer des périodes de demi-dissolution.

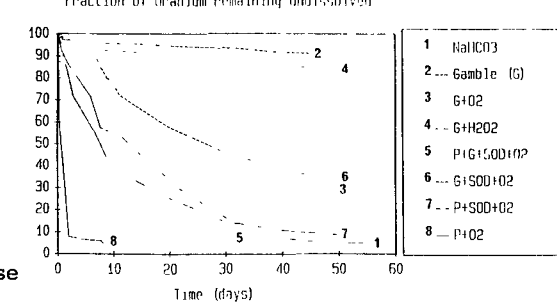
L'uranium est analysé par fluorimétrie.

Results

Le test statique ainsi que les différents solvants cités ont été étudiés sur les composés suivants : diuranates calcinés, UF_4 et UO_3.

. Uranates calcinés QML et NUFCOR : nous avons noté l'influence du barbotage d'O_2, équivalent à la solution de carbonates. QML (U_3O_8) a un comportement 20 % D et 80 % W alors que NUF (U_3O_8 + UO_x) est plutôt 40 % D et 60 % W.

. UF_4 : la figure 2 montre les résultats obtenus avec 8 solvants et l'importance de l'oxygène seul et des ions superoxydes qui entrainent une oxydation de U^{4+} en U^{6+}, augmentant la solubilité de ce composé. Celui-ci peut être considéré comme un composé de type D. Notons aussi une solubilité importante en milieu carbonates.



P = pyrogallol
SOD = superoxyde Dismutase

Figure 2 : Solubilité in vitro de l'UF_4 dans 8 solvants

. UO_3 : La solubilité de ce composé a été étudiée dans du BME, milieu plus riche que Gamble en protéines ainsi que dans d'autres solvants on constate qu'il a un temps de demi-dissolution inférieur à 10 jours. Nous avons observé un vieillissement de l'UO_3 séparé, dû à une hydratation dans le temps entrainant une solubilité différente (tableau 3).

TEMPS	U03 (extrait)	U03 (mélange)	U03 (hydraté)	U03 (four)
1j	17 % - 35 %	40 %	25 %	55 %
5j	28 % - 55 %	63 %	42 %	80 %

Tableau 3 = Résultats de solubilité dans le BME de 4 composés UO_3 avec des degrés hygrométriques variant exprimés en pourcentage d'uranium solubilisé.

Discussion

Les principales observations tirées de cette étude sont :

- l'insolubilisation de l'uranium quelque soit le composé en présence de phosphates et de sa grande solubilité en milieu carbonates,

- la solubilité augmentant avec le barbotage d'O_2 et donc un mécanisme d'oxydation des poussières.

- l'influence des ions superoxydes O_2^- surtout dans le cas d'UF_4, reproduisant ainsi un phénomène décrit au niveau du macrophage.

- la solubilité décroissante de la poussière d'UO_3 lorsque son degré d'hygrométrie augmente.

Tous ces résultats nous conduirons à essayer le BME comme solvant de base (comparable à Gamble mais dont la charge en protéines est supérieure) additionné d'oxydant.

III.3 - **Test in vitro cellulaire**

Ce test a été développé essentiellement en 1989 et semble très important puisqu'il se situe entre le test in vitro chimique et le test in vivo.

Methodology

Il s'agit d'un test in vitro sur macrophages alvéolaires de rats, maintenus en survie en phase gazeuse (5 % CO_2) dans un milieu de culture ou BME (fig. 4).

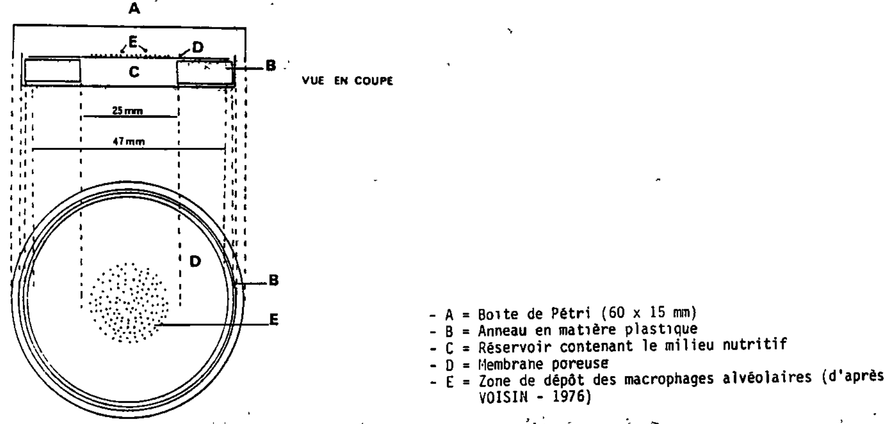


Figure n° 4 = Test in vitro cellulaire

Ce test est réalisé sur une période de 2 à 3 jours. L'activité des macrophages est suivie par un dosage d'Adénosine Triphosphate (ATP) intracellulaire. L'uranium aussi bien solubilisé que restant sur le filtre est analysé par fluorimétrie.

Results

Le composé principalement étudié a été l'UO_3 extrait du mélange industriel dont a granulométrie a été sélectionnée avec un AMAD de 3 µm. Ce test a mis en évidence un effet d'insolubilisation de l'UO_3 par les macrophages (fig. 5). Il a été réalisé sur les différentes formes d'UO_3 plus ou moins hydraté (tableau 6) : UO_3 industriel ou mélange de base, UO_3 extrait du mélange, UO_3 vieilli ou hydraté et UO_3 passé au four à 350 °C donc déshydraté.

CINETIQUE DE SOLUBILISATION D'UO3

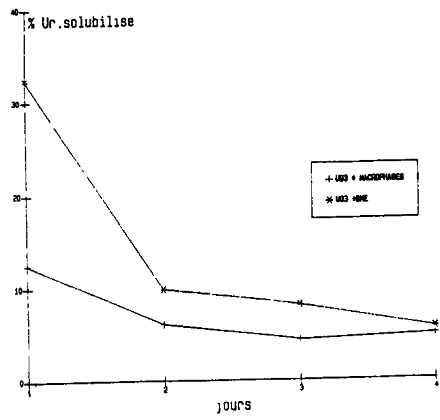


Figure 5 : UO_3 extrait du mélange

% DISSOUT LES PREMIERES 24 H	UO3 DESHYDRATE	UO3 EXTRAIT DU MELANGE	UHH	MELANGE INDUSTRIEL
SANS CELLULES	82,2 %	32,3 %	10,6 %	30 %
AVEC MACROPHAGES	69,8 %	12,5 %	9,8 %	25,5 %

Tableau 6 : Comparaison de la solubilité des différents composés UO_3 en présence ou en l'absence de macrophages alvéolaires de rats

Nous avons observé, comme pour les tests in vitro chimiques, la diminution de la solubilité avec l'augmentation de l'hydratation de la poussière.

Discussion

Les principales remarques concernant ces essais :

- Les macrophages semblent exercer un effet d'insolubilisation de l'UO_3 quelque soit son degré d'hydratation.

- L'augmentation du degré d'hydratation de l'UO_3 correspond à une diminution de la solubilité.

- Ce test in vitro cellulaire développé avec l'UO_3 devrait être poursuivi sur d'autres composés.

III.4 - **Etudes in vivo**

2 types d'études in vivo sur rats OFA ont été réalisés chronologiquement : dans un premier temps des études avec intoxication intratrachéale par les diuranates calcinés QML et NUFCOR, et dans un deuxième temps des études par inhalation par voie sèche d'UF_4 et d'UO_3.

Methodology

. Intoxication intratrachéale : les instillations ont été réalisées après anesthésie de l'animal après mise en suspension des sels d'uranium dans du NaCl. L'animal est ensuite placé en cage à métabolisme.

. Intoxication par inhalation : les rats sont placés dans une chambre d'inhalation sphérique de 8 l où la génération par voie sèche était assurée par un tube fritté : la poussière est déposée et soufflée avec un débit de 50 l mn^{-1}. Le temps d'exposition est de 20 mn et l'AMAD des poussières comprises entre 7 et 10 µm. (Il s'agit de poussières industrielles).

Quelques animaux sont sacrifiés juste après inhalation pour évaluer essentiellement la charge pulmonaire, et les autres sont placés dans des cages à métabolisme afin de suivre l'excrétion urinaire sur 15 à 20 jours. En fin d'expérimentation l'uranium est dosé dans les organes ou tissus suivants : poumons, reins, TGI, carcasse pour modéliser le transfert du composé.

Results

. Les résultats concernant l'intoxication intratrachéale ont montré des excrétions urinaires decroissantes avec pour NUFCOR un comportement de type D - W et pour QML, plutôt un comportement W confirmant les résultats observés in vitro.

. Les résultats concernant les inhalations sur UF_4 et UO_3 sont présentés pour UF_4 sous forme d'un tableau récapitulatif (tableau 7) et pour UO_3 nous ne présentons que la courbe d'excrétion urinaire moyenne (fig. 8) pour 11 rats.

TISSUE DISTRIBUTION OF URANIUM IN RATS AFTER INHALATION OF UF_4 % INITIAL LUNG BURDEN ($\bar{x} \pm$ SEM, N)					
DAYS	N	LUNGS	KIDNEYS	CARCASS	URINE
20	8	7.1 ± 0.6	0.4 ± 0.05	13 ± 2.1	43.9 ± 2.9

. SEM = standard Error of Mean

. N = number of animals per group

. Initial lung burden of uranium = 92 µg ± 9

($t_{1/2}$ = 5.2 d)

Tableau 7 : Tissue distribution of uranium in rats after inhalation of UF_4

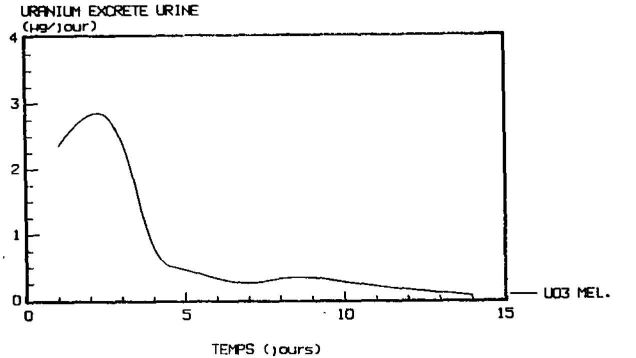


Figure 8 : Inhalation UO_3 mélange Malvesi
Excrétion urinaire journalière
Résultats moyens sur 11 rats

Discussion

. Nous avons observé que les excrétions urinaires étaient différentes entre l'intoxication intratrachéale et l'inhalation. Dans la première la décroissance de l'uranium est immédiate après une valeur forte à 24 H, alors que pour l'inhalation nous observons une bosse sur les 72 premières heures due à l'apport des poussières passant par le TGI.

La granulométrie utilisée dans les inhalations est importante (AMAD de 7 à 10 µm) et le compartiment principal est le naso-pharynx (NP). Ces conditions reproduisent le milieu industriel et correspondent à un cas peu étudié du modèle CIPR.

. L'UF_4 a montré un comportement de type D confirmant les essais in vitro chimiques avec une période d'environ 5 jours.

. Pour les composés industriels QML et NUFCOR on observe une bonne corrélation avec les résultats obtenus dans les tests in vitro.

. Les résultats concernant l'UO_3 sont en cours d'exploitation et semblent correspondre à un composé de type D - W.

III.5 - Etudes sur l'homme

Il s'agit de valider les méthodes et les stratégies de surveillance radiotoxicologiques de travailleurs exposés à des composés industriels d'uranium en France.

Methodology

- résultats radiotoxicologiques de personnels exposés (rétention pulmonaire et excrétions urinaires et fécales).

- résultats des contrôles atmosphériques journaliers (APA).

- Résultats des mesures des aérosols aux postes de travail (en particulier, diamètres aérodynamiques moyens).

Sur la base du modèle défini par la C.I.P.R. 30 et des données calculées par le Docteur PIECHOWSKI (DPS/SEAPS), le SHI a conçu un programme, implanté sur un microordinateur, permettant le calcul des incorporations soit lors d'une exposition chronique soit après une exposition unique, en tenant compte des caractéristiques du poste de travail et des résultats radiotoxicologiques.

Results

. Evolution des examens après exclusion d'un agent sur poste UO_2-U_3O_8 (fig. 9).

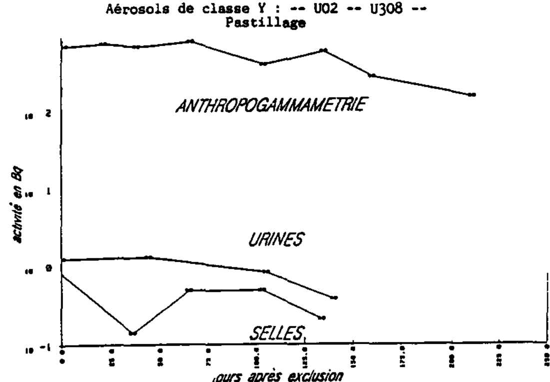


Figure 9 : Evolution des examens après exclusion

. Exposition à un oxyde d'uranium type U_3O_8 et calcul de la dose interne après incident.

Les hypothèses du calcul sont :

- exposition unique après incident,
- U_3O_8, composé de classe Y,
- diamètre aérodynamique moyen = 1 µm,
- composition isotopique = 234 U, 235 U, 238 U.

Les calculs sont effectués à partir des résultats observés sur les selles. Les doses effectives sont comprises entre 0.1 et 0.32 msivert.

. Suivi de l'exposition chronique à l'UF_4 entre 1977 et 1988 avec mesures des concentrations atmosphériques et de l'excrétion urinaire en parallèle présenté précédemment.

Discussion

. De nombreuses observations dans l'entreprise et notamment dans le cas de l'UF_4 ont permis de guider les expérimentations in vitro et in vivo.
En effet pour ce composé la décroissance rapide des urines suite à des incidents montrait qu'il avait plutôt un comportement D.

. Les résultats radiotoxicologiques permettent de calculer les équivalents de dose effective en chronique et également en cas d'incident.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

- DR. BEAU - DPS/SEGP - CEA - FONTENAY AUX ROSES
- Société COMURHEX - DR. GIBERT - MALVESI FRANCE
- Société FBFC - Dr. BOURDEIX - ROMANS FRANCE
- M. METIVIER - CEN/FAR - FONTENAY AUX ROSES FRANCE

V. Publications:

1 - E. Ansoborlo, J. Chalabreysse, E. Escallon
Etude de l'influence de différents paramètres sur la solubilité in vitro de composés industriels ou diuranates d'ammonium calcinés. Radioprotection 1989 - Vol 24 n° 1 pp. 3-12

2 - B. Gibert, E. Ansoborlo, J. Chalabreysse
Exposition chronique aux composés uranifères : problèmes médicaux de surveillance liés à leurs propriétés physico-chimiques et à leur solubilité - Arch. Mal. Prof., 1989, 50, n°6, pp. 582-591.

3 - M.H. Hengé-Napoli, E. Rongier, E. Ansoborlo, J. Chalabreysse
Comparison of the in vitro and in vivo dissolution rates of two diuranates and research on an early urinary indicator of renal failure in humans and animals poisoned with uranium - Radiation Protection Dosimetry - 1989 - Vol. 26 - pp. 113-117.

4 - E. Ansoborlo, P. Bérard and J. Chalabreysse
Study of industrial exposure to class Y uranium compounds : Methods and results - Radiation Protection dosimetry 1989 - Vol. 26 - pp. 101-105

5 - E. Ansoborlo, J. Chalabreysse, S. Escallon, MH. Hengé-Napoli
"In vitro solubility of uranium tetrafluoride with oxidizing medium compared to in vivo solubility in rats" - publication en cours pour IJRB 90.

6 - F. Bourdeix, P. Bérard, J. Achiary
La surveillance individuelle de l'exposition interne aux oxydes d'uranium - Radioprotection 89 - vol. 24 n° 3 p. 181-189

7 - J. Chalabreysse, P. Beau, etc...
Expérience acquise en France sur la radiotoxicologie des composés industriels de l'uranium - Radioprotection 89 - Vol. 24 - n° 3 - p. 191-202.

8 - Beau P.G., Chalabreysse J. -
"Mise en évidence par le retour d'expérience, de particularités métaboliques et toxicologiques de l'UF6 et de ses produits de dégradation". Workshop - Biological Assessment of occupational exposure to actinides - Versailles - 30 mai - 2 juin - 1988.

9 - Hengé-Napoli M.H., Rongier E., Chalabreysse J.
Suivi de la Gamma GT urinaire après intoxication au nitrate d'uranyle chez le rat - Communication aux 3èmes Journées Scientifiques de suze la Rousse, les 16, 17, 18 septembre 1987.

10 - VERDERA J.M.
Contribution à l'étude de la solubilisation des oxydes d'uranium - Ecole Centrale Paris - Thèse soutenue le 29 juin 1987.

11 - J. Camarasa - J. Chalabreysse - R. Bertrand - E. Ansoborlo
About work locals pollution by uranium bearing dust - 1st international Symposium on ventilation for contaminant control - Toronto - Canada - Octobre 85.

Title of the project no.:

Etude de la toxicité réelle des composés de l'Uranium en situation industrielle.

Head(s) of project:

Dr. J. CHALABREYSSE.

Scientific staff:

E. ANSOBORLO - M. ARCHIMBAUD - P. BERARD - M.H. HENGE-NAPOLI - E. PUJOL.

I. Objectives of the project:

Evaluer les risques réels, les caractéristiques physico-chimiques et les comportements métaboliques des composés d'uranium produits ou traités dans l'industrie nucléaire.

II. Objectives for the reporting period:

Les résultats obtenus in vivo chez l'homme et l'animal ont mis en évidence pour l'UF_4 une excrétion urinaire rapide caractéristique d'un composé D.

Dans ce cadre les essais in vitro chimiques ont été poursuivis en étudiant de nouveaux solvants entrainant des mécanismes de dissolution équivalent à ceux constatés in vivo. De même nous avons développé des essais in vitro cellulaires sur macrophages alvéolaires de rats, et des études in vivo par inhalation sur rats avec suivi des excrétions urinaires ainsi que sacrifice et analyse des organes et tissus.

III. Progress achieved:

III.1. Etudes in vitro

Les études de solubilité in vitro ont pour but de déterminer l'appartenance des composés industriels d'uranium (plus particulièrement UF_4 et UO_3) aux classes D, W et Y de la CIPR.

Les tests utilisés sont de 2 types : 1 test in vitro chimique et 1 test in vitro cellulaire sur macrophages alvéolaires de rats.

Méthodology

a) Test in vitro chimique

Nous avons poursuivi l'étude de liquides de solubilisation afin de se rapprocher le plus possible d'une part des résultats des tests in vitro cellulaires et d'autre part des phénomènes observés in vivo (animal, homme), en utilisant un test statique défini dans nos études précédentes.

Les solvants retenus pour ce test ont été successivement :

. Solution de bicarbonate
. Solution de Gamble avec barbotage d'oxygène
. Solution de Gamble avec addition de Pyrogallol et de Super oxyde dismutase (SOD) sous barbotage d'oxygène (formation d'ions superoxydes O_2^-)
. Milieu de culture cellulaire (BME) sous 5 % de CO_2.

Ces test sont réalisés en température à 37°C. Les cinétiques observées sont exprimées sous forme d'exponentielles permettant de calculer des périodes de demi dissolution.

b) Test in vitro cellulaire

Un test sur macrophages alvéolaires de rat maintenus en survie en phase gazeuse (5% CO_2) a été appliqué à l'étude de la solubilisation de poussières uranifères (UO_3).

En bref, la description du test est la suivante : une rondelle en matière plastique, évidée en son centre de façon à créer un puits, est placée dans une boite de Pétri. Le puits est rempli de milieu nutritif BME. Une

membrane poreuse recouvre ce puits. Les cellules et la poussière à tester sont déposées à la surface du puits à raison de 10^6 cellules par boite et d'environ 1 particule par cellule.

Pour les témoins, on ne dépose que la poussière, sans cellules.
La quantité d'uranium solubilisée en 24 heures est dosée dans le milieu nutritif du puits.

Le test est réalisé sur une période de 2 à 3 jours. L'activité des macrophages est suivie par un dosage d'Adenosine-triphosphate (ATP) intracellullaire.

Dans ces 2 tests l'Uranium solubilisé ou restant sur filtre est dosé par fluorimétrie.

Results

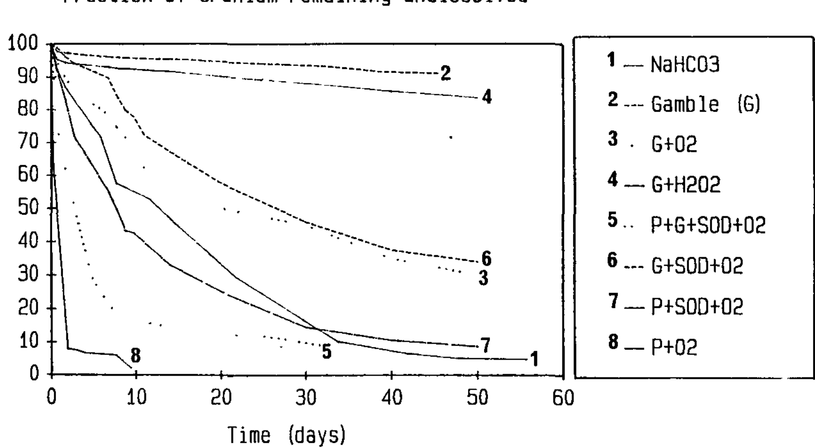
a) Test in vitro chimique

C'est avec UF4 que l'on a comparé l'effet des différents solvants.

Nous avons cherché à reproduire le phénomène d'oxydation auquel on assiste dans l'étude de solubilisation avec le macrophage. Avec la solution de Gamble + barbotage d'oxygène et la solution de Gamble + Pyrogallol et superoxyde Dismutase, on assiste à un phénomène d'oxydation de la poussière.

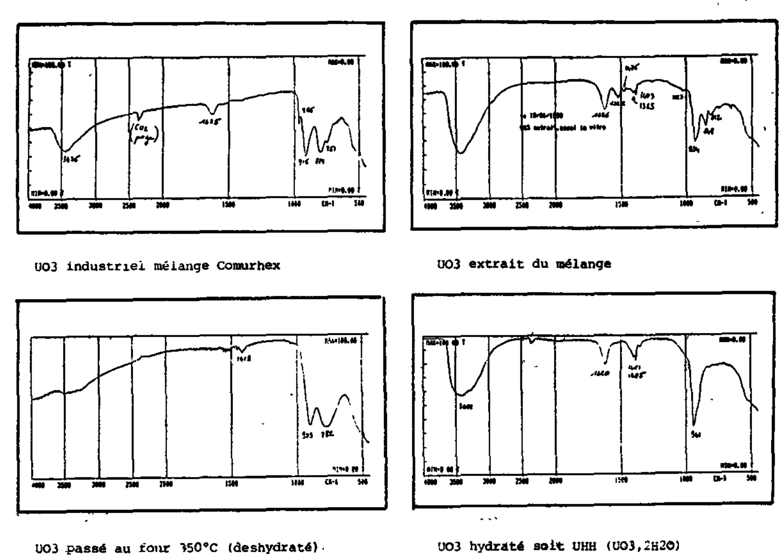
Ces résultats sont présentés dans la courbe 1 et ont montré un passage de U4+ en U6+ sous forme ion uranyle entrainant une solubilité rapide de ce composé de type D avec une période de l'ordre de 2 à 3 jours.

FIG. 1



Dans le cas d'U03 industriel nous avons dans un premier temps procédé à une caractérisation physico-chimique du composé et séparé celui-ci en 3 constituants principaux : le Diuranate d'Ammonium (ADU < 5 %), le sesquioxyde d'Uranium U308 (U308 < 5 %) et l'U03.

Nous avons constaté un vieillissement à l'air de l'U03 séparé dans le temps du à un phénomène d'hydratation de la poussière mis en évidence par des analyses en Infrarouge solide (Fig.2). Ce degré d'hydratation variable de l'U03 joue un grand rôle dans la solubilité de ce composé (Tableau 3) dans un milieu de culture cellulaire (BME).



U03 industriel mélange Comurhex

U03 extrait du mélange

U03 passé au four 350°C (deshydraté)

U03 hydraté soit UHH (U03,2H20)

Fig 2 : SPECTRES INFRA ROUGE DE 4 TYPES U03.

TEMPS	U03 (extrait)	U03 (mélange)	U03 (hydraté)	U03 (four)
1j	17 % - 35 %	40 %	25 %	55 %
5j	28 % - 55 %	63 %	42 %	80 %

Tableau 3 = Résultats de solubilité dans le BME de 4 composés UO_3 avec des degrés hygrométriques variant.

Le tableau 4, présente la solubilité de U03 extrait et U03 industriel dans divers milieux.

t	U03 industriel			U03 séparé du mélange		
JOURS	Gamble +02	Gamble+P+SOD	HCO_3^-	Gamble +02	Gamble+P+SOD	HCO_3^-
1	81,5 %	84,8 %	54,8 %	65,3 %	78,2 %	44,9 %
5	57,8 %	62,1 %	5,2 %	40,8 %	54,9 %	4,7 %
10	41,5 %	50,1 %	0,5 %	30,9 %	43,1 %	0,6 %
30	17,9 %	27,2 %	/	5,2 %	14,9 %	/

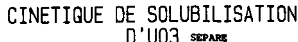
P = Pyrogallol
SOD = Super Oxyde Dismutase

TABLEAU 4 = Solubilité in vitro chimique de U03 industriel et U03 extrait de ce mélange dans divers milieux.
Résultats exprimés en pourcentage cumulé restant non solubilisé au temps t.

b) Test in vitro cellulaire

Le composé principalement étudié a été l'U03 extrait du mélange industriel dont la granulométrie a été sélectionnée avec un AMAD retenu de 3 µm.

Ce test a mis en évidence un effet d'insolubilisation de l'U03 par les macrophages (Fig.5). Il a été réalisé sur les différentes formes d'U03 (Tableau 6) à savoir : U03 industriel ou mélange de base, U03 séparé de ce mélange, U03 vieilli ou hydraté (UHH) et U03 vieilli deshydraté au four à 350°C.



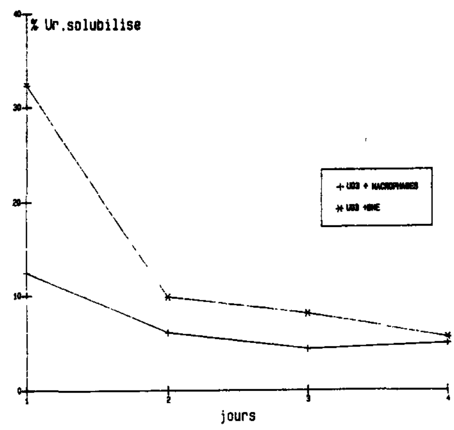


Fig 5 U03 EXTRAIT DU MELANGE

% DISSOUT LES PREMIERES 24 H	UO3 DESHYDRATE	UO3 EXTRAIT DU MELANGE	UHH	MELANGE INDUSTRIEL
SANS CELLULES	82,2 %	32,3 %	10,6 %	30 %
AVEC MACROPHAGES	69,8 %	12,5 %	9,8 %	25,5 %

Tableau 6 : Comparaison de la solubilité des différents composés en présence ou en l'absence de macrophages alvéolaires de rats

Nous avons observé comme pour les tests chimiques l'influence du degré d'hydratation sur la solubilité.

Discussion

- La connaissance des caractéristiques physico-chimiques de la poussière étudiée est essentielle et a permis de mettre en évidence d'une part 3 composés dans l'UO3 industriel de base et un phénomène d'hydratation de l'UO3 extrait.

- Cette hydratation de l'UO3 a une influence importante sur la solubilité aussi bien dans les tests chimiques que cellulaires. Plus la poussière est hydratée moins elle est soluble.

- Les macrophages exercent un effet d'insolubilisation de l'UO3 quelque soit son degré d'hydratation.

- Dans l'ensemble l'UO3 sous ses différentes formes semble plutôt avoir une solubilité rapide avec un temps de demi-dissolution inférieur à 10 jours avec des périodes différentes.

Pour l'UF4 nous constatons que le solvant permettant de reproduire les mécanismes de dissolution équivalent à ceux constatés in vivo sont Gamble + Pyrogallol + Superoxyde dismutase sous barbotage O2 ; Pour l'UO3 c'est le BME.

Ces 2 résultats nous conduirons à essayer le BME comme solvant de base (comparable à Gamble mais dont la charge en protéine est supérieure) additionné d'oxydant.

III.2. Etudes in vivo

Objectif

Etude in vivo par inhalation de l'U03 industriel et comparaison des cinétiques de solubilisation in vivo et in vitro.

Méthodology

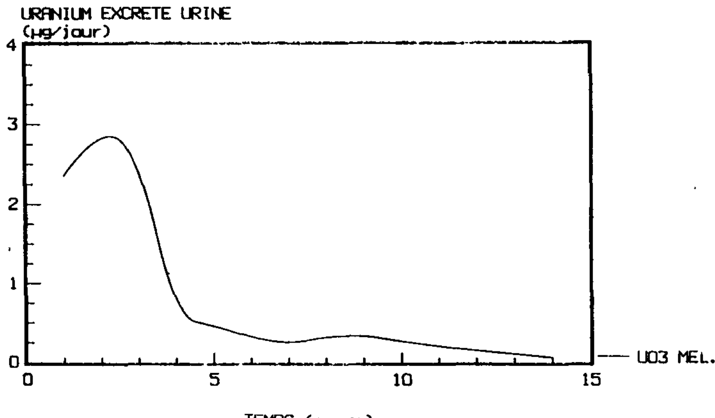
Expérimentation sur rats (OFA) avec génération de poussières sèches. Le diamètre aérodynamique moyen est de 8 µm avec une déviation standard de 2. 2 expérimentations on été réalisées: Dès la première nous avons suivi l'excrétion urinaire des rats (12 rats) et au bout de 14 jours nous avons sacrifié tous les animaux et procédé au dosage de l'uranium des différents organes : carcasse, ensemble trachee poumon, reins, TGI.

Results

Les résultats de la première expérimentation sont regroupés dans le tableau 7 et l'excrétion urinaire moyenne sur les 12 rats a été représentée dans la fig. 8.

ORGANES / t	POUMONS (TB + P)	REINS	TGI	CARCASSE	URINES
14 j	1,15 µg	0,09 µg	4,4 µg	10 µg	10,7 µg

Tableau 7 : Résultats d'analyses d'organes en Uranium suite à une inhalation d'U03 industriel après 14 jours.



De même par la seconde inhalation sur 6 rats les résultats sont donnés dans le tableau 9.

ORGANES / t	POUMONS (TB +P)	REINS	TGI	CARCASSE	URINES	TETE	PEAU TETE	SANG
Sacrifice immédiat t = 0	2,36 µg	/	314 µg	214 µg	/	52 µg	734 µg	/
t = 1 j	0,85 µg	/	1157 µg	56 µg	1,26 µg	2,7 µg	50 µg	/

Tableau 9 : Résultats d'analyses d'organes en uranium suite à la seconde inhalation d'UO3 industriel. Sacrifice immédiat et à 24 h exprimé en µg dans l'organe.

Discussion

La granulométrie de ces 2 expériences est importante : l'AMAD est de 8 µm correspondant à un dépôt principal dans le Naso Pharynx (NP) (83 %), confirmé dans la deuxième expérimentation ou l'analyse de la tête comprenant à la fois la bouche et le NP donc une valeur très supérieure à celle du poumon, ce qui correspond à la réalité industrielle.

L'élimination urinaire principale se fait sur les 3 premiers jours alors que dans le modèle CIPR, qu'il s'agisse d'un composé D ou W cette même élimination devrait se faire en 24 heures.

Les résultats sont en cours d'exploitation et semblent correspondre à un composé de type D - W.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

- Dr. BEAU - DPS/SEGP - CEA - Fontenay aux Roses
- Société Comurhex - Dr. GIBERT - Malvesi
- M. METIVIER - IPSN - Fontenay aux Roses

V. Publications:

1 - E. ANSOBORLO - J. CHALABREYSSE - S. ESCALLON
Etude de l'influence de différents paramètres sur la solubilité in vitro de composés industriels ou diuranates d'ammonium calcinés.
Radioprotection 1989 - Vol. 24 n°1 pp. 3 - 12.

2 - B. GIBERT - E. ANSOBORLO - J. CHALABREYSSE
Exposition chronique aux composés uranifères : problèmes médicaux de surveillance liés à leurs propriétés physico-chimiques et à leur solubilité - Arc. Mal. Prof., 1989, 50, n°6, pp. 582-591.

3 - M.H. HENGE-NAPOLI - E. RONGIER - E. ANSOBORLO - J. CHALABREYSSE
Comparison of the in vitro and in vivo dissolution rates of two diuranates and research on an early urinary indicator of renal failure in humans and animals poisoned with uranium Radiation Protection - Dosimetry 1989 - Vol. 26, pp. 113-117.

4 - E. ANSOBORLO - P. BERARD and J. CHALABREYSSE
Study of industrial exposure to class Y uranium compounds : Methods and results.
Radiation Protection dosimetry 1989 - Vol 26, pp. 101-105.

5 - E. ANSOBORLO - J. CHALABREYSSE - S. ESCALLON - M.H. HENGE-NAPOLI
In vitro solubility of uranium tetrafluoride with oxidizing medium compared to in vivo solubility in rats.
Publication en cours par IJRB 90.

6 - F. BOURDEIX - P. BERARD - J. ACHIARY
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7 - J. CHALABREYSSE - P. BEAU - etc ...
Expérience acquise en France sur la radiotoxicologie des composés industriels de l'uranium.
Radioprotection 89 - Vol. 24 n°3, pp. 191-202.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-076-UK

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Title of the research contract:

Local retention and translocation of particles in the respiratory tract.

List of projects:

1. Mechanisms governing particle translocation and related aspects of lung function.
2. Spatial distribution of particles in the lung in relation to cells at risk.

Title of the project no.: 1

Mechanisms governing particle translocation and related aspects of lung function

Head(s) of project: Dr G Patrick

Scientific staff: Dr G Patrick

I. Objectives of the project:

The primary aim was to clarify those cellular and physiological processes which determine the movement of particles within the respiratory tract, especially to sites which are important in lung dosimetry. This is of value for the development of improved dosimetric models to provide a more realistic assessment of the risk of lung cancer from inhaled radioactive particles. The approach was to be based mainly on methods for the selective deposition of particles in specific regions of the respiratory tract of experimental animals.

II. Objectives for the reporting period:

The above aims were to be pursued by three different methodologies:

(a) tracheobronchial retention of particles deposited on the conducting airways, administered by the intratracheal instillation of test particles;

(b) tracheobronchial retention of particles administered by inhalation;

(c) alveolar clearance mechanisms of particles administered by microinjection.

III. Progress achieved:

(a) <u>Tracheobronchial retention of particles: intratracheal instillation</u>

Methodology

Rats of the Fischer-344 strain were administered $^{133}BaSO_4$ test particles via an intratracheal cannula, using techniques developed previously in this laboratory. The volume of suspension instilled was only 2 μl, to achieve local deposition in the distal trachea. The particles had a count median diameter of 0.34 μm with a geometric standard deviation of 1.7. The instillation was made 10 min after recovery from anaesthesia so that muco-ciliary clearance was not impaired by the anaesthetic. Clearance was measured by whole-body gamma counting, including scans along the length of the animal, and by serial sacrifice at intervals up to 24 weeks after instillation (3).

To ascertain the pattern of lymphatic drainage in the rats used, colloidal carbon was injected intraperitoneally, intratracheally (at a volume of 0.5 ml to ensure deposition throughout the lungs) and by micro-injection (2-7 μl) into the interstitial tissue of the tracheal wall. Lymph nodes were assessed for carbon content by light microscope examination of histological sections (4).

Results

The amount of ^{133}Ba remaining in the distal trachea after one week, where the particles had been deposited, was 0.41% of the initial dose. By 24 weeks the amount had decreased to 0.10%, and the clearance curve from 1 to 24 weeks could be satisfactorily fitted by a single exponential term with a half-time of 88 days. These figures were derived from serial killing and radioassay of the distal trachea (2,3).

Similar clearance rates were calculated from whole-body gamma counting. The scanning profile of ^{133}Ba along the thorax did not change between 1 and 24 weeks (3).

In a related study the pathways of lymphatic drainage in the thorax of the Fischer rat were defined, using colloidal carbon as a tracer material. It was shown that while the lung lobes were drained mainly to the posterior mediastinal lymph nodes, the tracheal wall was drained primarily to the internal jugular and posterior cervical nodes (4).

Following the instillation of $^{133}BaSO_4$ particles into the distal trachea, the amount of ^{133}Ba found in the internal jugular and posterior cervical lymph nodes increased to a maximum of 0.022% of the injected dose by 8 weeks, and slowly decreased thereafter (3).

Discussion

The retention of $^{133}BaSO_4$ particles in the distal trachea after 1 week was similar to values reported earlier for a variety of types and sizes of particle. Measurement of the long-term clearance from this region had not been attempted before. It was clear that particles retained in the airway wall were removed only slowly, at a rate not very different from alveolar clearance during the same period. For this reason, when particles are inhaled and clearance is followed by external thorax counting, the slow clearance of particles from the walls of the conducting airways is obscured by the retention and clearance of the much larger amounts deposited in the alveolar region (7).

Some of the particles cleared from the tracheal wall were removed to the lymph nodes which normally drain that region. However it is not clear what proportion of the deposit was cleared by that route.

(b) Tracheobronchial retention of particles: inhaled particles

Methodology

A new approach has been investigated to see if it possible to analyse retention in the rat trachea following the administration of particles by inhalation. For this purpose it is necessary to undertake a metabolic study of excretion of cleared particles, and to use highly insoluble test particles.

In an exploratory study Fischer rats have inhaled (nose-only) 4 μm (median aerodynamic diameter) monodisperse fused aluminosilicate particles (FAP) labelled with ^{57}Co. The large diameter ensured a relatively high deposition in the large airways. The rats were whole-body counted at intervals up to 28 days, and were housed in metabolism cages during the periods 4-7 and 21-28 days. At sacrifice after 0, 7 and 28 days the trachea was dissected free and its lumen washed repeatedly with saline. A measured length of washed trachea was assayed for ^{57}Co, as were the tracheal washes.

Results

The washing procedure removed 99% of the particles from the trachea in rats killed immediately after inhalation. After 28 days only 85% could be removed by washing in the same way. The tracheal wall contained 0.016% of the total body burden at that time.

By measuring the rate of urinary and faecal excretion of ^{57}Co, it was possible to calculate the rate at which ^{57}Co-FAP was being removed from the lung by "mechanical" clearance - i.e. clearance as particles - to the gastro-intestinal tract, and by dissolution and translocation to blood. By assuming that the velocity of muco-ciliary clearance up the trachea of the rat is 5 mm/min, in accordance with an earlier estimate in this laboratory, and from the measured length of the washed segment of trachea, it was possible to calculate the amount of ^{57}Co-FAP which was being cleared through this segment by muco-ciliary clearance. Results so far indicate that this amount comprised no more than 5% of the ^{57}Co that was washed from the trachea at 28 days.

Discussion

This study has suggested that it is possible to analyse the particle content of the trachea of the rat into three compartments: particles being rapidly cleared (by muco-ciliary action), particles on the surface of the trachea but not being rapidly cleared, and particles incorporated into the airway wall. This has been achieved following administration by inhalation, and may therefore be more readily compared with studies on delayed clearance reported elsewhere, including human volunteer studies.

(c) <u>Alveolar clearance mechanisms</u>

Methodology

Particles of colloidal gold have been injected into subpleural alveoli of rat lung, using a glass micropipette with a bevelled tip of outside diameter 10-14 μm (1). The colloid was prepared by reduction of $H^{195}AuCl_4$ with ascorbic acid, to produce polydisperse ^{195}Au-labelled gold particles with a count median diameter of 15-30 nm. It was confirmed by gel chromatography that the colloidal preparation contained no soluble (ionic) gold.

The micropipette was passed into the left lung through the parietal pleura, from a small area of which the overlying muscle had been removed. This technique ensured the deposition of particles within 1-2 mm laterally and most of the particles were found on immediate sacrifice to be in the alveoli adjacent to the pulmonary pleura (1). The alveoli were not flooded, since the volume of colloidal suspension injected was typically 0.05 μl; the mass of particles injected was not greater than 1 μg. Removal of the micropipette did not collapse the lung. After injection the animals were allowed to recover from the anaesthetic.

20 rats were injected for short-term studies on the fate of the gold particles. Later a long-term study using 38 rats was commenced, using the same techniques. After injection and periodically throughout the study the rats were whole-body counted, and at certain intervals the excretion of ^{195}Au was assessed using metabolism cages. Rats were sacrificed immediately and after intervals of 1 day, 1 week and 1, 4, 9 and 15 months, for dissection and radioassay. The left lung was divided along its length into 2-mm slices, and those containing most ^{195}Au into 2-mm cubes, for assay and for autoradiography and electron microscopy.

Results

The short-term studies up to 24 h after injection showed that there was no rapid clearance of particles from the alveoli. Very little gold was removed by muco-ciliary clearance to the extrapulmonary airways and gastro-intestinal tract. Also, the tiny amounts found in the liver and spleen up to 24 h indicated that very little of the ^{195}Au had reached the blood; the kidney content and overall excretion level also confirmed that there had been no significant dissolution of the particles.

By 4 min after injection a small proportion of the gold particles had already been taken up by alveolar macrophages (AM) in the deposition region. Phagocytosis by AM was well under way by 1 h, when particles were seen on the cell surface, in phagolysosomes and in the cytoplasm. After 5 h nearly all the particles were within AM: by this time many of them were no longer confined to the deposition region, but had spread more evenly throughout the alveolar tissue up to a few hundred μm beneath the pleural surface. There were still a few particles free on the alveolar septa. By 5 h a few macrophages containing gold particles were seen at the terminal bronchioles, both on the ciliated epithelium and close to the peribronchiolar tissue adjoining the neighbouring alveoli. Other AM

containing gold were seen around blood vessels. No particles were seen within lung cells other than AM, and none had penetrated to the interstitium (5,6).

Most of the work on the long-term study has been completed. All of the animals have been killed, the last group at 15 months after injection. In this group the whole-body retention data for 6 rats has been obtained for the full period. It was confirmed, as noted above, that there was no rapid early clearance phase of gold particles from the alveoli. The mean lung burden, relative to the initial value, was 94% at 1 week after injection, 87% after 2 weeks, but was still 48% at the end of the experiment. Interestingly, the range of values among the 6 rats was large, corresponding to 27-69% after 15 months.

On dissection it was found that, of the ^{195}Au retained in the body, more than 90% remained in the left lung at all times after injection. By 9 months 2.6% of the remaining body burden was in the thoracic lymph nodes, as compared with 2.2% in the liver, 0.02% in the spleen and 0.50% in the kidneys.

The pattern of excretion at different times was analysed, and showed for example that from the 4th to the 8th week after injection the percentage of total ^{195}Au excretion found in the urine fell from 5.3 to 1.1%. Supplementary experiments have also been conducted to define the excretion pattern of soluble ^{195}Au injected i.v. into rats: the results will be used to analyse the overall metabolism of the injected gold colloid and thereby to define the true rate of "mechanical" clearance of particles from the alveoli.

Radioassay of the slices of left lung showed that, as before (1), the initial deposit was confined to within 1-2 mm of the deposition site. This relative pattern did not change appreciably over the whole period of the study. The pattern of ^{195}Au distribution among the 2-mm cubes of fixed tissue taken from the slices with most activity in them changed to some extent, but the greater part of the particles remained close to the original site of injection; only a small fraction was seen at locations between there and the main axial bronchus, and around the hilum.

The autoradiographic and electron microscopic examination of the large number of sections of lung taken at the different time points is still being completed. Preliminary results from this part of the work present some interesting findings. The large proportion of gold particles retained close to the injection site is within AM, which often form

aggregates of cells near the pleura. From 1 day after injection through to 15 months, ^{195}Au has been seen on the surface of the ciliated airways; from 1 week onwards this was observed right up to the hilum. After 1 week gold was also observed in the tissue around the airways and from 1 month around airway bifurcations. At 4 months after injection particles were noted in lymphoid tissue near the hilum. The cellular location of these deposits remains to be clarified by electron microscopy.

Discussion

The data from the short-term studies confirm that the microinjection technique results in a pattern of deposition where nearly all of the particles are in sub-pleural alveoli, with a small proportion in alveoli a few hundred μm deeper into the lung, but with no deposition on any of the ciliated airways. Hence it is possible by this technique to study clearance specifically from the alveolar region of the lung and to study the movement of particles resolved over quite small distances within the lung.

While there is no rapid component to the kinetics of alveolar clearance, the lung retention curves do not fit a single exponential curve. Most of the clearance process is well described, however, by an exponential term with a mean half-time of some 500-600 days. This average value should be interpreted with caution, in view of the wide variation between animals in this study. Since they were all injected at about the same time, with the same preparation of gold colloid and otherwise under very similar conditions, this variation may reflect differences in the location of the injection site in relation to the micro-architecture of the lung. The effect of such differences could have been detected because of the very small volume of lung into which most of the particles had been injected.

Even at long times after injection there was clearly no widespread redistribution of particles throughout the lung. In principle, AM are capable of bringing about such a redistribution, for example by lateral movement between alveoli through the pores of Kohn. Instead there is only a small degree of redistribution to the tissue surrounding the conducting airways, which may represent the lymphatic drainage pathway, reaching to the hilum and including lymphoid aggregates in that region. Other than that, the injected particles seem either to leave the lung altogether by

AM movement and muco-ciliary clearance, or else to remain in AM close to the original site of deposition.

When the final analyses are completed, these findings, together with those described in the second project, will have important consequences for the future development of dosimetric lung models (7). It is necessary for these purposes to understand how, and to what extent, particles deposited in alveolar lung or on the conducting airways can be retained long-term at sites near to cells at risk of lung cancer induction.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

V. Publications:

1. Patrick G & Stirling C (1986) "A method for microinjection into subpleural alveoli of rat lung in situ", J Appl Physiol 60, 307-310.

2. Takahashi S & Patrick G (1986) "Long-term retention of $^{133}BaSO_4$ particles in the rat trachea", Proc 4th Int Congr Toxicol, Tokyo.

3. Takahashi S & Patrick G (1987) "Long-term retention of ^{133}Ba in the rat trachea following local administration as barium sulfate particles", Radiat Res. 110, 321-328.

4. Takahashi S & Patrick G (1987) "Patterns of lymphatic drainage to individual thoracic and cervical lymph nodes in the rat", Lab Animals 21, 31-34.

5. Patrick G & Stirling C (1987) "The study of alveolar macrophages in rat lung by direct microinjection through the pleura", EULEP Newsletter 45, 37-40.

6. Patrick G & Stirling C (1988) "The clearance of particles of colloidal gold from subpleural alveoli", Annals Occup Hygiene 32, suppl. 1, 1164-1166.

7. Patrick G (1989) "Requirements for local dosimetry and risk evaluation in inhomogeneously irradiated lung", in Low Dose Radiation - Biological Bases of Risk Assessment, eds K F Baverstock & J W Stather, pp 269-277, Taylor & Francis, London.

Title of the project no.: 2

Spatial distribution of particles in the lung in relation to cells at risk.

Head(s) of project: Dr G Patrick

Scientific staff: Dr A L Batchelor
Dr K J Morris

I. Objectives of the project:

The aim was to make a quantitative assessment of the spatial distribution of particles retained in the lung at long times after inhalation. Using human lung, measurements were to be made of the distances of occupationally inhaled particles from defined target cells, e.g. in the airway epithelium. This would permit an assessment to be made of the dose to the target cells in the lung from alpha-emitting particulates. Parallel studies were also to be made in the rat to study the spatial distribution of particles with respect to the airways up to two years after inhalation.

II. Objectives for the reporting period:

The above aims were followed by four main lines of enquiry:

(a) particle distribution in human lung;
(b) clearance from rat lung of inhaled tourmaline particles;
(c) UO_2 distribution in rat lung;
(d) cellular effects of inhaled UO_2.

III. Progress achieved:

1. Methodology

(a) Particle distribution in human lung. Post mortem specimens of lung were obtained in the first instance from three Cornish tin miners. They had been exposed occupationally in the mines to dust from the local granite rock which was known to contain sufficient boron to allow its detection in histological lung sections. This was due to the presence in the rock of the mineral tourmaline, which is a crystalline complex silicate that occurs in many granites and contains approx. 3% boron. It can therefore be detected by neutron-induced autoradiography (NIAR) on CR-39 solid state track detector fashioned as microscope slides.

5 µm sections were cut from different regions of the lung specimens, after resin-embedding, and mounted on CR-39 slides. The resin was removed from the sections with sodium ethoxide. Alternate sections were placed on standard glass slides for conventional histology. A second pre-etched CR-39 slide was placed tightly over each section mounted on CR-39, and the pairs of slides were exposed to thermal neutrons in a reactor to approx. 10^{12} cm^{-2} (1). This produced 1.5 MeV alpha-particles from the tourmaline by the ^{10}B(n,alpha) reaction, which were recorded in the CR-39 as short tracks. The top CR-39 slide was removed for etching in 40% KOH and track counting. The bottom slide with the section on it was re-exposed to a total of approx. 10^{15} neutrons cm^{-2}, to generate dense clusters of tracks. An image of the lung tissue itself was then produced on the bottom slide of CR-39 by exposure to ^{238}Pu alpha-particles attenuated in energy. The tracks from the tourmaline and the tissue image were rendered visible by etching the CR-39 with 40% KOH. At this stage the tissue section itself was lost. The dense clusters of tracks from boron in the tourmaline were clearly visible against the tissue image, so enabling them to be located in the lung tissue. The smaller numbers of tracks on the top slide, which were readily counted, could then be related to their corresponding position on the bottom slide (1).

(b) Clearance from rat lung of inhaled tourmaline particles. To make some validation that tourmaline particles might be a suitable model for inhaled actinides in the lung, an experiment was conducted in which an aerosol of tourmaline was inhaled by rats and its clearance compared with that of PuO_2 and other particles in the rat.

Cornish tourmaline was obtained and ground to a powder. The quartz content was reduced to approx. 5% by differential sedimentation. Further wet grinding in a micronizing mill (McCrone) reduced the particle size to the respirable range. The prepared tourmaline was then neutron-activated to give a radioactive gamma-emitting label suitable for measuring retention in the rat lung: the most useful label was ^{54}Mn, produced from chemically-bound iron in the mineral. After irradiation the tourmaline was examined by X-ray diffraction (4).

Fischer-344 rats were exposed nose-only to the activated tourmaline aerosol at a concentration of approx. 20 mg m^{-3}. The particle size was determined using a 7-stage cascade impactor. The rats were assayed for ^{54}Mn in a small animal whole-body counter after 3 days. They were sacrificed in groups of four at intervals from 3 to 180 days after inhalation. The mass of tourmaline retained in the lungs was determined from the ^{54}Mn activity, also the liver content in order to estimate dissolution or leaching of the label from the tourmaline. ^{54}Mn was measured in these samples with a Ge(Li) detector (4).

(c) UO_2 distribution in rat lung. This study was undertaken to be complementary to the distribution work on human lung. 50 Fischer-344 rats were exposed nose-only to an aerosol of UO_2 at a concentration of 111-220 mg m^{-3}. The particle size was measured as in (b). The UO_2 was obtained enriched to 93% ^{235}U and thereby also to 0.8% ^{234}U which provided most of the alpha dose to the lung. The rats were assayed for UO_2 in the whole-body counter after 5 days. They were sacrificed in groups of five at intervals from 180 to 720 days after inhalation, with additional animals at times from 1 to 90 days.

The lungs were fixed in inflation by intratracheal instillation of OsO_4 in fluorocarbon and each lobe was cut into 3-mm slices, further fixed in glutaraldehyde and resin-embedded for neutron-induced autoradiography (NIAR, see below). Other tissues were assayed for UO_2 in the same rats by delayed neutron analysis. Additional animals were sacrificed at intervals from 5 to 630 days for similar UO_2 analysis of the lungs .

The autoradiographs have been examined by light microscopy and image analysis (Seescan). Clusters of small numbers of tracks were counted manually, larger ones assessed by area on the image analyser. Each field of view scanned was also analysed for the area of each of the major tissue types in the lung, so that UO_2 concentrations per unit area could be

calculated as a measure of the volume particle concentration.

In the course of developing the methodology of NIAR with CR-39 (see (a) above), it was found that at higher neutron fluxes an image of the tissue structure was formed on the top slide, which had been in close contact with the tissue section itself during irradiation in the reactor (1). Under the right conditions the image was adequate for determining the location of particles in the lung, e.g. whether on alveolar septa or associated with airways or blood vessels. The nature of this image was such that it was not possible to distinguish from it the small tracks due to boron-containing material, but it was certainly possible to distinguish it from the much larger fission fragment tracks e.g. from ^{235}U. The advantage of this procedure was that, unlike the former method using alpha-particles to produce a tissue image on the bottom slide (a), the tissue section itself on the bottom slide was not lost, and could be used for direct histological comparison, instead of using an adjacent section.

It was considered that the image formed on the top slide might have been caused by the natural boron content of the tissue. Therefore the technique was further developed whereby the tissue section was soaked in 10% $NaBO_2$ for 1 h. After a brief rinse and dehydration, a top slide of CR-39 was placed tightly over the section mounted on the bottom slide, and the pair was exposed to approx. 10^{14} neutrons cm^{-2}. After etching in KOH as before, the top slide was used for microscopic examination both of fission fragment tracks from UO_2 particles in the lung and of the associated tissue image (2). It was also observed that useful images could be readily obtained from other tissues, e.g. kidney and brain.

(d) Cellular effects of inhaled UO_2. As part of a study on the effects of neutrons and UO_2 on the lung, and of fission fragments induced by neutrons after inhalation of UO_2, groups of four HMT rats were exposed to an aerosol of ^{235}U-enriched UO_2 similar to that in (c), with or without subsequent exposure to neutrons. The rats were sacrificed 8 days after inhalation and samples of lung tissue prepared for transmission electron microscopy (TEM) by standard techniques. The morphometry of alveolar macrophages, type I and type II epithelial cells and blood leucocytes was studied using a Quantimet-720 image analyser (3).

2. Results

(a) Particle distribution in human lung. For the amounts of boron found in the miners' lungs, the neutron fluences used here produced adequate numbers of tracks for locating particles in the lung sections. The tracks produced by the ^{10}B(n,alpha) reaction were very short, appearing after etching with KOH as small pits. The dense clusters of these pits, produced by 10^{15} neutrons cm^{-2} on the bottom CR-39 slide, overlapped and were readily visible as large pits contrasting well with the tissue image produced as described above. From this it was possible to identify the location of tourmaline particles, or aggregates of particles e.g. within macrophages, relative to the tissue structure. Confirmation of the structure was possible from the alternate sections prepared for conventional histology (1).

From the lung sections of the three available cases, a study of the location of the tourmaline in the lung showed deposits around the walls of small bronchioles; some of these appeared to be in the lamina propria, within 40 µm of the ciliated epithelium, but most would be described as peribronchiolar and were further away from the epithelium (5).

A similar study of control lung tissue, obtained from subjects who had not been occupationally exposed to rock dust, showed no detectable boron by this technique.

(b) Clearance from rat lung of inhaled tourmaline particles. Examination of the neutron-activated tourmaline by X-ray diffraction showed that it had retained its crystalline structure, although the diffraction peaks were slightly broadened.

The mass median aerodynamic diameter (MMAD) of the aerosol was 3.3 µm, and the geometric standard deviation (GSD) 2.0. The mean lung burden at 3 days was 24 µg (4).

Over the period 3-90 days the lung retention data could be fitted by a single exponential term with a slope (λ) = 1.50×10^{-2} $days^{-1}$, which was useful for comparison purposes. For PuO_2, mixed Pu and U oxides, and Fe_2O_3, data obtained elsewhere over the same period could be fitted by single exponentials with $\lambda = 1.22\text{-}1.65 \times 10^{-2}$ $days^{-1}$.

The ^{54}Mn content of the rat liver measured at 7-90 days was used to estimate the rate of dissolution or leaching of the radionuclide from the particles. An upper estimate for this was 0.36×10^{-2} $days^{-1}$, indicating that tourmaline is indeed highly insoluble in the lung; the true rate of

"mechanical" clearance of tourmaline particles from the lung would then be between 1.14 and 1.50 x 10^{-2} days^{-1}. In any case, this was not significantly different from the values for the other aerosols quoted above (4).

(c) UO_2 distribution in rat lung. The UO_2 aerosol generated for this study had a MMAD of 2.7-3.2 and a GSD of 1.7. The mean initial lung burden was 270 μg. The analysis of the mass of UO_2 in the different organs confirmed the highly insoluble nature of this aerosol: 81% of the body burden was in the lungs after 720 days. 10% was in the thoracic lymph nodes, 0.8% in the kidneys and 3.9% in the skeleton. The average tissue concentration in the lungs fell from 173 μg g^{-1} after 5-11 days to 21 μg g^{-1} after 630 days, but the concentration in the lymph nodes reached less than 11 μg g^{-1} (6).

The lung content after 720 days represented 17% of the initial lung burden at 5 days. Interestingly the clearance curve over the whole period could be adequately described by a single exponential term, where the rate constant λ = 0.32 x 10^{-2} days^{-1} (half-time = 219 days). By 720 days the accumulated mean lung alpha dose was estimated to be 5.7 Gy. By 540 days, when the dose was 5.2 Gy, very little fibrosis or other pathological effects were seen in the lung sections. After 720 days, however, fibrosis, metaplasia and some tumours were found (6).

All the autoradiographs of the lung sections have been prepared, and the analysis of the UO_2 distribution is nearing completion. Each deposit of UO_2 identified under the microscope has been allocated to one of the following categories: alveoli, alveoli within 100 μm of an airway, pleural/subpleural, perivascular, airway, airway bifurcation, malignant diseased tissue, non-malignant diseased tissue.

(d) Cellular effects of inhaled UO_2. The mean lung burden 6 days after inhalation was 140 μg UO_2, giving a mean lung alpha dose to sacrifice at 8 days of 0.01 Gy. The UO_2 significantly increased the size of alveolar macrophages and also that of type II epithelial cells; the number of macrophages and of type I cells were increased. The lysosomal granules in the macrophages were also increased in size. Exposure to neutrons and to fission fragments had no significant effect on any of the cells studied beyond those observed in animals exposed to UO_2 alone (3).

3. Discussion

(a) Particle distribution in human lung. Work done on the limited number of specimens made available showed that it was possible to locate deposits of boron-containing mineral dust such as tourmaline. Their distribution in the lung could be ascertained with reference to the lung tissue image, and the mass of each deposit could be assessed by track counting.

Thus tourmaline could be a useful material for assessing the long-term spatial distribution of particles in man, since a population exists which inhales sufficient quantities to be detectable by the techniques described here. At the same time these lungs could be obtained free of overt lung disease, e.g. silicosis. Morever, tourmaline is a very insoluble material. It may therefore serve as model for describing the long-term spatial distribution of accidentally inhaled insoluble particles of actinide compounds such as PuO_2 (see also (b) below). This would permit an assessment to be made of the accumulated alpha dose from inhaled actinides to cells at risk from lung cancer induction, e.g. cells in the epithelium of the conducting airways. It should be noted that examination of accident cases themselves, e.g. involving PuO_2 inhalation, would be unlikely to give sufficient information because too few particles would be detectable in histological sections and determination of their spatial distribution would be impractical.

No more specimens of tin miners' lung became available during this study. It was concluded that the data available did not permit a proper analysis of the spatial distribution of such particles, as more specimens would have been required. Nevertheless, it was shown that tourmaline particles can be retained in human lung such that a proportion of them are within 40 μm of the epithelium of the conducting airways (5). This means that, if tourmaline is a good model for inhaled alpha-emitting particulates such as PuO_2, then alpha-irradiation of target cells in the epithelium in human lung is possible from particles retained long-term.

(b) Clearance from rat lung of inhaled tourmaline particles. Tourmaline clearance could not be measured by its boron content, since there was more boron in control rat lungs than the amount deposited as tourmaline. For this reason neutron-activated tourmaline was prepared as described above.

The tourmaline particles were cleared from rat lung at a similar rate to PuO_2 and mixed Pu and U oxides, where these actinides had been administered at low levels such that the alpha-irradiation of the lung would

not impair clearance. The rate was also similar to that for Fe_2O_3, which is non-toxic in the lung. Therefore it can be concluded that, as regards the rate of clearance from the lung, tourmaline is a good model for actinides of interest in practical radiological protection, and indeed for other less toxic aerosols. In so far as the period 3-90 days after the inhalation of rather insoluble particles represents a phase of clearance that is dominated by the action of alveolar macrophages, it can be assumed that tourmaline at the levels employed here does not impair macrophage function (4).

This by itself does not prove that the spatial distribution of tourmaline in human lung will correspond exactly to that of the insoluble actinides retained long-term, but it does support the use of tourmaline in distribution studies.

(c) UO_2 distribution in rat lung. This study was a continuation of previous work which described the UO_2 distribution 2-30 days after inhalation. Analysis of the distribution of the same type of particle is now being completed up to 2 years after inhalation, which will indicate the relative doses from long-term retention to the different regions of lung, e.g. the conducting airways, vascular tissue, the pleura, etc.

This study has been made possible by the associated development of techniques for NIAR and image analysis of the autoradiographs. The use of boronated tissue to provide an image on the CR-39 top slide proved to be better than could be achieved with non-boronated sections, and at lower neutron fluxes. The chief advantage over the previous method of imaging the lung onto the bottom CR-39 slide is that the section itself is not lost by the etching procedure, and so is available for histological examination.

(d) Cellular effects of inhaled UO_2. The UO_2 particles used in these studies clearly produced early as well as late effects on the lung. The effects on alveolar macrophages seen at 8 days after inhalation may have been associated with the slower rate of clearance of these particles, as compared with e.g. low levels of inhaled PuO_2. The inhibition of clearance may have been due to a combination of chemical toxicity, alpha dose, and the moderately high initial lung burden of the aerosol (3).

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

Harwell Laboratory, UK Atomic Energy Authority, Oxon. UK;

Department of Physics, University of Bristol, UK: Dr D L Henshaw;

Atomic Weapons Establishment, Aldermaston, Berkshire, UK;

British Geological Survey, Keyworth, Nottingham, UK: Dr J K Ball.

V. Publications:

1. Morris K M & Batchelor A L (1987) "The location of boron-containing dust in the lung, utilising neutron-induced autoradiography techniques with a CR-39 solid state track detector", Phys. Med. Biol 32, 1501-1508.

2. Morris K M & Batchelor A L (1988) "The simultaneous imaging of boronated tissue sections and the location of fissionable actinide particles in CR-39 solid state track detector, utilising a neutron-induced autoradiographic technique", Phys. Med. Biol 33, 1195-1203.

3. Morris K M, Townsend K M S & Batchelor A L (1989) "Studies of alveolar cell morphometry and mass clearance in the rat lung following inhalation of an enriched uranium dioxide aerosol", Radiation & Environmental Biophysics 28, 141-154.

4. Batchelor A L (1989) "Clearance of inhaled tourmaline rock dust from the rat lung", J Aerosol Sci 20, 639-645.

5. Patrick G (1989) "Requirements for local dosimetry and risk evaluation in inhomogeneously irradiated lung", in Low Dose Radiation - Biological Bases of Risk Assessment, eds K F Baverstock & J W Stather, pp 269-277, Taylor & Francis, London.

6. Morris K J, Khanna P & Batchelor A L "Long term clearance of insoluble enriched uranium dioxide particles from the lung of the Fischer-344 rat", Health Physics, in press.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-078-F

Fondation Bergonié
Cours de l'Argonne, 229
F-33076 Bordeaux Cédex

Head(s) of research team(s) [name(s) and address(es)]:

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Title of the research contract:

Mechanism of radiation-induced leukemogenesis and osteosarcomagenesis.

List of projects:

1. Mechanism of radiation-induced leukemogenesis and osteosarcomagenesis.

Title of the project no.:

B16-D-078 F

Head(s) of project:

E. LEGRAND, B. GUILLEMAIN

Scientific staff:

T. ASTIER-GIN, H. BAYLAC-KALABOKIAS
Technicians : 2

I. Objectives of the project:

The genome of some B-ecotropic recombinant retrovirus which emerge during the process of radiation-induced leukemogenesis of the C57BL/6 mouse may be suspected to act as the causative factor or as a cofactor in this process. To test for this hypothesis we looked for new retroviral integration sites in the DNA of primary and transplanted tumors. Thirty per cent such tumors were found to harbor a limited number of new retrovirus insertions. The role of these viruses was evaluated by analyzing the cell populations present in primary and corresponding *in vivo* propagated tumors and by investigating their pathologic potential.

II. Objectives for the reporting period:

To investigate the leukemogenic potential of new ecotropic recombinant proviruses encountered in radio-induced thymic lymphomas of the C57BL/6 mice. To correlate biological properties of such viruses with their genomic organization.

III. Progress achieved:

1.- Methodology

The new ecotropic provirus encountered in a radio-induced thymic lymphoma of the C57BL/6 mouse was molecularly cloned. The *env* and LTR regions were sequenced and compared to other prototype retroviruses. The viral DNA was used to transfect murine cells in order to evaluated its ability to replicate and to assess its leukemogenic potential.

2.- Results and discussion

We previously reported that new ecotropic recombinant proviral sequences were found to be clonally integrated in a significant number (30 %) of primary or transplanted radio-induced tumors of the C57BL/6 mouse. Such proviral sequences were already detected in *in vivo* or *in vitro* propagated radio-induced thymoma cells (Jolicoeur et al., 1983) but none of the viral isolates derived from these cells was found to be highly leukemogenic. We wondered whether in our tumors one could detect ecotropic proviral sequences markedly different of the endogenous ecotropic one and eventually associated with the outgrowth of the tumor. For this we screened 31 primary or transplanted (few passaged) radio-induced lymphomas for the presence of new ecotropic proviruses with either an anormally long LTR or with a specific integration site. Noteworthingly, such a proviral sequence was found but in only one tumor. The provirus with an anormally long LTR was inserted in the chromosomal DNA within the PVT-1/MLVi-1/Mis-1 region which is a common integration site for MCF virus in mice and for Mo-MuLV in rats. This new ecotropic provirus was then molecularly cloned and found to be infectious and competent for replication after transfection of murine cells. The recovered virus termed **T3651/B** was shown to be B-ecotropic and of XC^{+} phenotype. T3651/B virus is T-lymphotropic (*in vivo*) and highly leukemogenic for newborn C57BL/6 mice. It is also highly leukemogenic for adult animals provided they are submitted to a subleukemogenic dose of irradiation. Our results indicate that although all CD4/CD8 cell populations of the newborn are potential targets for the neoplastic transformation by T3651/B virus, such cell populations in

the unirradiated adult animals are resistant. In contrast, the $CD4^+/CD8^+$ populations of the irradiated adult mice are the preferential target for neoplastic transformation. These observations are in accordance with the hypothesis proposed several times, of a radioresistant T-cell subpopulation(s) raised by irradiation and becoming the target(s) for radio-induced retroviruses. Although we cannot say that T3651/B virus emergence was the primary event leading to tumorigenesis because it was isolated at the 4th *in vivo* passage of 3651 tumor cells, it remains that it is a new thymotropic highly leukemogenic virus with the ability to induce thymic lymphomas as irradiation does.

Rassart et al. (1986) investigated the molecular events leading to the emergence of RadLVs starting from the parental endogenous ecotropic non leukemogenic C57BL provirus and ending to the passaged highly leukemogenic RadLV-VL_3. In their model, they proposed that after irradiation, the first variants which appear are fibrotropic non leukemogenic B tropic MuLVs which arise via a gag-pol recombination between endogenous ecotropic and xenotropic proviruses. In a second step, few modifications (base substitutions) arising in the LTR and the appearance of a C rich sequence just in front of the "TATA" box confer to the B-tropic fibrotropic virus a weak leukemogenic potential. Next, the thymotropism and a higher leukemogenic potential are acquired by additional base changes within *env* and U3 LTR and more importantly by a 43 bp direct repeat within U3.

The two viruses cloned and sequenced in our investigation (T1223/B and T3651/B) are interesting elements which could be situated in the upper model. The weakly leukemogenic B-ecotropic T1223/B isolated from the RadLV/Rs complex was shown to have a xenotropic-like gag-pol region and to have base changes within *env* and U3. In contrast to the B-tropic weakly leukemogenic primary RadLVs isolated by Rassart et al., T1223/B virus LTR does not harbor any C rich sequence upstream of the "TATA" box ; however, this virus has a base substitution in U3 enhancer inducing the appearance of a consensus sequence for the nuclear factor SEF1, a protein highly expressed in T lymphocytes which binds to the SL3-3 virus enhancer. Therefore the T1223/B virus enters in the same class as the primary weakly leukemogenic RadLVs Ti.8 and Ti.9 of the

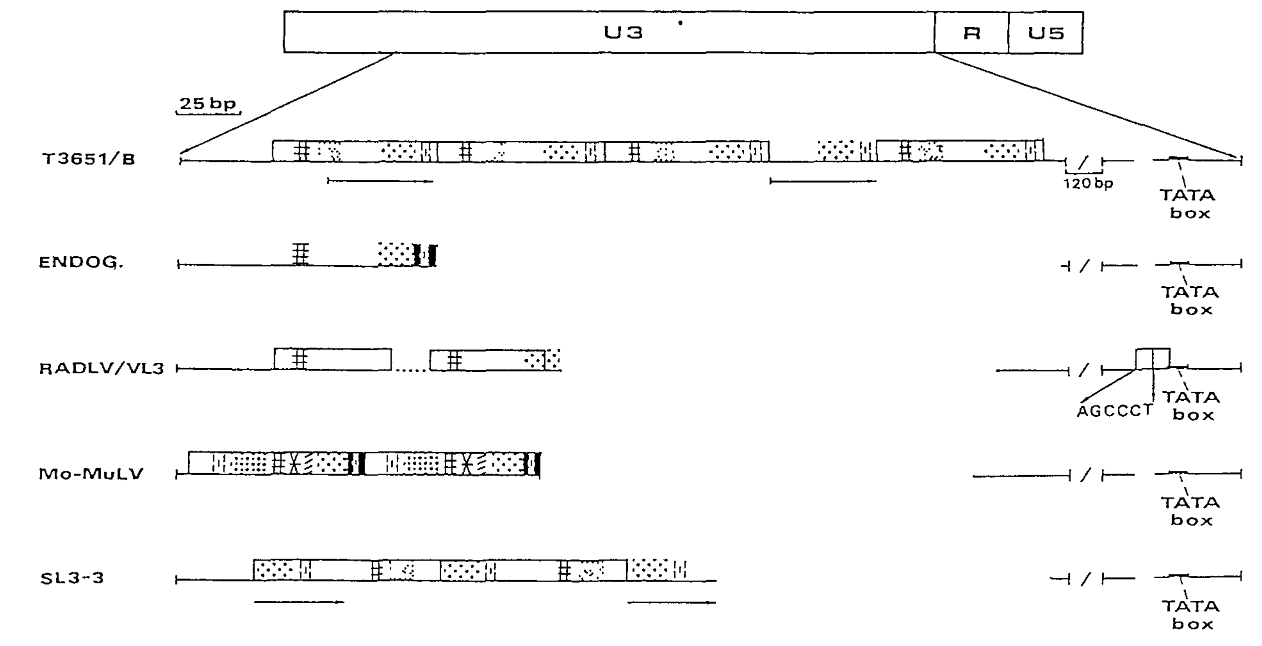
model. Although, the nature of the *env* and LTR modifications which may be associated to a weak leukemogenic potential is unclear, it is interesting to remark that two different LTR alterations may lead to such a property.

With respect to its leukemogenic potential, the T3651/B virus compares well with the cloned RadLVs isolated by Jolicoeur et al. (1986) and Merregaert et al. (1987) from the Kaplan's strain of RadLV, BL/VL_3. Both types of viruses have important modifications in the U3 region of their LTRs consisting in long tandem direct repeats of enhancer sequences. However, these repeats differ between T3651/B and the clones isolated from the RadLV/VL_3 by the lenght and the number of repeats as well as by the nature and number of sequence motifs specifically recognized by nuclear factors. Indeed T3651/B LTR displays 5 tandem repeats. The 3 formers are perfect repeats of a 64 bp strecht of an endogenous U3 proviral sequence (from nucleotide 136 to nucleotide 199) while the fourth has only the last forty bases (159-199) and the fifth has one substitution (G $\longrightarrow$ A position 426) and a deletion of the last base pair (position 430). Besides, if one considers the inference of substitutions and/or repetitions on the appearance of consensus sequences for nuclear factors, the T3651/B virus LTR has 4 LVb, 4 SEF1, 5 NF1-like and 4 LVa consensus sequences.

It seemed important to compare this LTR structure of T3651/B virus to those of other thymotropic highly leukemogenic MuLV. The figure is a schematic representation of the respective configurations of U3 sequences including the direct repeats of T3651/B, C57BL/Ka endogenous provirus, RadLV/VL_3, Mo-MuLV and SL3-3. Interestingly the 4 leukemogenic viruses have common features. Indeed, in contrast to the C57BL endogenous ecotropic provirus, they harbor direct repeats of the same U3 region containing enhancer sequences with specific binding motifs for nuclear factors. The presence of several consensus sequences for nuclear binding factors and their repetitions in the LTR of these viruses allow to speculate that they are the major regulatory elements which lead to T-cell specific expression and leukemogenicity. This is supported in particular by the data brought by Thornell et al. (1988) who showed that the nuclear factor SEF1 is predominantly

expressed in T-cell lineages and could be an essential factor for the T-cell tropism of SL3-3 MuLV and the ability of this virus to cause T-cell lymphomas. This could also apply to T3651/B virus which displays repetitions of the same consensus sequence for the SEF1 factor. Although RadLV/VL_3 has a less complex U3 general organization, it is noteworthy that it has numerous mutations along the LTR and particularly a C-rich sequence containing a 6 bp direct repeat located in U3 just in front of the promotor "TATA" box. Such modifications can also be implicated in the thymoma induction ability of this virus.

Finally, two thymotropic highly leukemogenic retroviruses, namely RadLV/VL_3 and T3651/B, have been isolated after the irradiation of C57BL mice. Both induce thymic lymphomas, originate from endogenous retroviral sequences and have LTRs with repeats of enhancer sequences. However, these LTRs differ by the number and nature of the specific nuclear factors which can bind to them. It is thus possible that the mechanism implied in cell neoplastic transformation are not strictly identical for the two viruses.



Schematic representation of U3 sequences including the direct repeats of T3651/B virus, C57BL/Ka endogenous provirus, RadLV/VL_3, Mo-MuLV and SL3-3 viruses. Repeats are shown as boxes or horizontal arrows. In order to save space, scale is changed for 120 bp upstream the TATA box. For a better alignment of homologous regions, different gaps were introduced in the five U3 schemes. Dots in RadLV/VL_3 scheme indicate a 11 bp insertion. Consensus motifs specific of the nuclear factors are noted as follows LVb, SEF1, NF1-like, LVa, GRE, core binding factor, LVc, NF1

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

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Abteilung für Pathologie der Gesellschaft dür Strahlen und Umwelftorschund, Neuherberg, Germany

V. Publications:

H. BAYLAC-KALABOKIAS, T. ASTIER-GIN, B. BORREMANS, E. LEGRAND, R. HOOGHE, M.P. HOUBEN-DEFRESNE, M. JANOWSKI, J.F. DUPLAN and B. GUILLEMAIN -
Evidence of recombinant ecotropic provirus integration in thymic lymphomas induced by direct or indirect radiation effects.
Leukemia Research, **13**, 131-143, 1989.

D. VAILLIER, R. DACULSI, E. LEGRAND and B. GUILLEMAIN -
Production of cytokines after lipopolysaccharide stimulation of spleen cells during lymphoma development in AKR mice.
Cancer Immunol. Immunother., **29**, 35-42, 1989.

T. ASTIER-GIN, E. LEGRAND, H. BAYLAC-KALABOKIAS and B. GUILLEMAIN -
Identification of malignant cell clones in radio-induced murine thymic lymphomas by viral and cellular probes.
Leukemia (accepté).

D. VAILLIER, R. DACULSI, E. LEGRAND and B. GUILLEMAIN -
NK Activity, production of interferon and interleukin 2 in immunodeficient C57BL/6 mice injected with RadLV-Rs viral complex.
Immunobiology (accepté).

R. DACULSI, E. LEGRAND, D. VAILLIER, J.F. DUPLAN and B. GUILLEMAIN -
Effect of interleukins and anti-CD3 monoclonal antibody on the proliferation of radioresistant murine thymocytes.
Thymus (soumis).

H. BAYLAC-KALABOKIAS, T. ASTIER-GIN, D. MOYNET, M. HERNOULD, R. MAMOUN, E. LEGRAND and B. GUILLEMAIN -
A new leukemogenic retrovirus isolated from tumor cells derived from a radio-induced of the C57BL/6 mice : Analysis of the *env* and LTR sequences.
J. Virol. (soumis).

RADIATION PROTECTION PROGRAMME
Final Report

Contractor:

Contract no.: BI6-D-080-D

Gesellschaft für Strahlen-
und Umweltforschung mbH
Ingolstädter Landstr. 1
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Head(s) of research team(s) [name(s) and address(es)]:

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Telephone number: 089-3187 2312

Title of the research contract:

Pathogenesis of late somatic effects of radiation.

List of projects:

1. Radiation-induced oncogenesis under different exposure conditions.
2. Pathogenesis of radiation-induced cancer.

Title of the project no.: 1

Radiation-induced oncogenesis under different exposure conditions

Head(s) of project:

W. Gössner

Scientific staff:

W. A. Müller, U. Linzner, A. Luz, A. B. Murray, E. Schäffer

I. Objectives of the project:

The aim of this project is to study the modification of dose dependence in radiation-induced oncogenesis paying particular attention to the effects of different qualities of radiation, radiation dose rates and time patterns, injection mode, and age dependence. In addition the combined application of two different α-emitting radionuclides has been studied.

II. Objectives for the reporting period:

a) The influence of an irradiation free interval during internal irradiation of bone by short-lived bone-seeking radionuclides.
b) The effect of dose protraction with a very low ^{224}Ra activity in mice.
c) Conditions of local osteosarcoma induction and distribution and dosimetric studies with the local applied radiothoriumcolloids ^{227}Th, ^{228}Th.
d) The influence of age at incorporation of short-lived bone-seeking radionuclides.
e) Combination experiments with simultaneous incorporation of two different radionuclides.

III. Progress achieved:

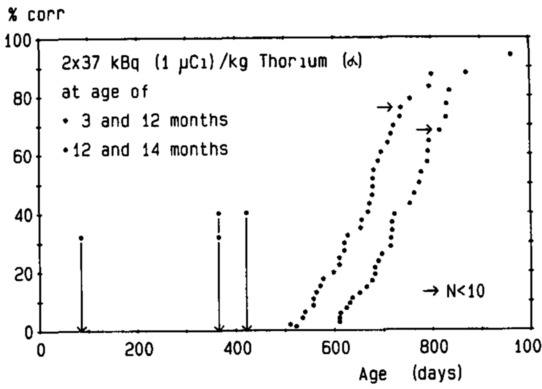
1. Methodology

In experiments investigating systemic effects the radionuclides were injected intraperitoneally as isotonic solutions.
Female NMRI/Nhg mice from the GSF breeding colony were used.
During the long-term experiment mice were checked on 5 days of the week.
A complete autopsy was performed on all animals found dead or killed with obvious neoplasia or in moribund state. Macroscopic diagnoses were confirmed by histology.
Osteosarcoma incidence was corrected for competing risks according to Miescher et al. (*Schweiz. Med. Wschr. 71:1002-1007, 1941*).

2. Results

a) The influence of an irradiation free interval. Comparison of short-lived α- and β-emitters.

In order to study special time patterns 37 kBq/kg of the short-lived (half-life 18.7 days) α-emitter ^{227}Thorium were incorporated twice: At age of 3 and 12 months or 12 and 14 months (Fig. 1a). The earlier incorporation of one half of the activity in the first group induced a shortening of the latency time as compared to the second experimental group. This means that the activity incorporated at 3 months of age was additive to the activity incorporated at 12 months despite the irradiation free interval.



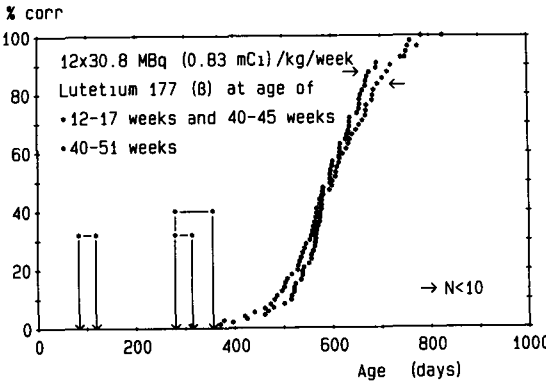


Fig. 1 a + b
Corrected osteosarcoma incidence after fractionated internal irradiation.
Female NMRI-mice.
Incorporation schedule indicated within diagrams.
a) internal α-irradiation by incorporation of ^{227}Thorium
b) internal β-irradiation by incorporation of ^{177}Lutetium

A corresponding experiment with the short-lived (half-life 6.7 days) β-emitter ^{177}Lutetium showed a contrasting result (Fig. 1b). 12 weekly incorporations of 8 MBq/kg were applied continuously during 40 to 51 weeks of age or in portions (of 6 injections) at age 12-17 weeks and 40-45 weeks. In both groups cases with osteosarcoma occurred at the same age. This means that the first injection series during weeks 12-17 lost some part of its effectiveness during the irradiation free interval.

b) Dose protraction with a very low ^{224}Ra activity

Two groups of approximately 300 4-week-old female NMRI-mice were used in the experiment. One group received a single injection of 18.5 kBq/kg ^{224}Ra body weight, corresponding to a mean skeletal α-dose of 0.15 Gy. The other group received the same total amount of ^{224}Ra in the form of 72 fractions of 257 Bq/kg each, applied twice weekly over a period of 36 weeks. This scheme resulted in a mean skeletal dose rate of about 1 mGy/day, and the same final mean total skeletal dose of 0.15 Gy (s. also Table I). The unexpected result observed in this experiment was the early induction of malignant lymphoma in the protracted group. Figure 2 shows the situation up to the end of the experiment, corrected for competing risks. The final number (percentage) of lymphomas was nearly equal for all experimental groups, but the time-course was different: In the protracted group a relatively greater number of lymphomas occurred very early, starting during the injection period and stopping shortly after the injections ceased. In contrast, in the second half of life lymphoma incidence showed a comparable slope to that in the other groups. Only the protracted irradiation group showed a considerable number of lymphomas in the first year of life.

The single injection group showed a later onset of lymphomas than the controls in spite of the fact that there were three times more animals in this group than in controls.

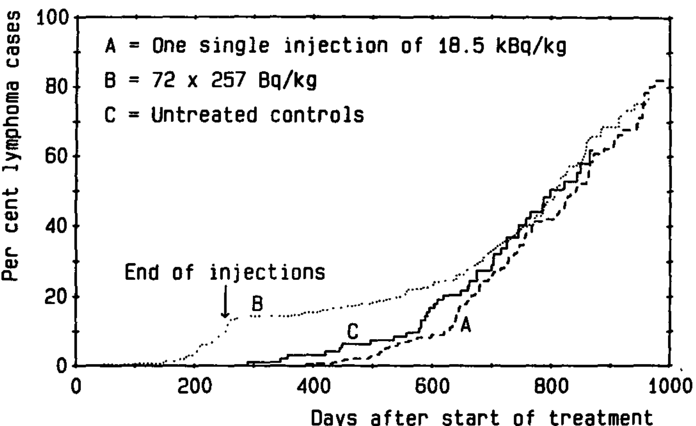


Fig. 2 Incidence of lymphomas after single and protracted injections

Table I Dosimetry data and induction of osteosarcomas after i.p. injection of ^{224}Ra

Group	Treatment	Total skeletal dose Gy	Skeletal dose rate mGy/d	Osteosarcomas whole life % (cases/total number of animals
A	Single injection 18.5 kBq/kg	0 15	60.00 [a)]	5 8 % (17/295)
B	Protracted injections 72x257 Bq/kg	0 15	1 13 [b)]	7 0 % (21/299)
C	Untreated controls	0	0	3 0 % (3/99)

[a)] maximum dose rate shortly after injection [b)] mean dose rate during injection span

The histopathology showed that in the first period up to about day 300 all lymphomas were of the lymphoblastic type, whereas after this period mixed types of lymphomas were also observed in all three experimental groups (Müller, Linzner & Luz, 1988).
After day 600 all three groups showed a very similar induction of lymphomas reaching an absolute percentage between 40 and 50 % (see Fig. 2).
The cumulative incidence of osteosarcoma induction is shown in Fig. 3.
There was a similar increase in the single and protracted group up to day 800. After this period osteosarcomas were observed only in the protracted group with seven cases out of 21 animals surviving beyond day 800. The percentages in Fig. 3 are corrected for competings risks as in Fig. 2, and were stopped when the number of surviving animals was less than ten in order to avoid an exaggerated evaluation of the last osteosarcoma cases. The total numbers of osteosarcomas and absolute percentages are given in Table I together with the dosimetric data. There was no statistically significant difference in the unadjusted total percentages of osteosarcomas in the two treated groups.

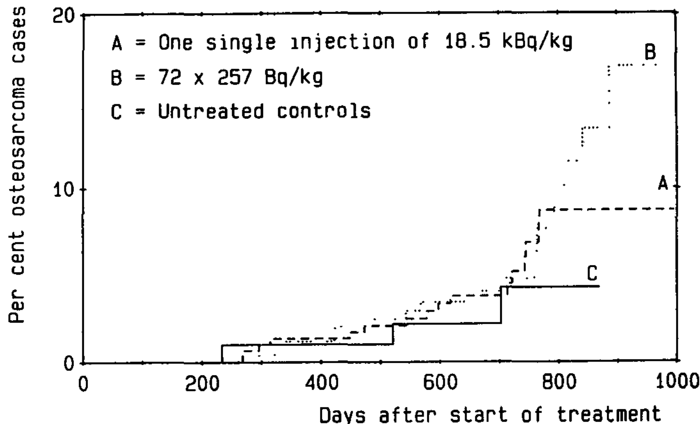


Fig. 3 Incidence of osteosarcomas after single and protracted injections

c) Local osteosarcomas

Former experiments have shown that ^{144}Cerium radiocolloid can induce a high yield of local osteosarcomas in Sprague-Dawley rats after parosteal administration. Experiments with NMRI-mice with one fifth of the ^{144}Ce activity of the rat experiment showed analogous results with high local osteosarcoma rates. Experiments with shorter-lived β-emitting radiocolloids did not induce local osteosarcoma, not even with multiple injections.

In experiments with radiothoriumcolloids it could be shown that 95 % of the thorium isotopes stayed at the injection site whereas the radium daughters were distributed throughout the skeleton in a similar way as after i.p. injected radium (s. Fig. 4).

The first results of the pilot studies showed a high systemic (anywhere in the skeleton outside of the injection site) incidence of osteosarcomas, i.e. ca. 50 % for 9 kBq ^{227}Th per mouse or 3 kBq ^{228}Th per mouse, and a few local osteosarcomas in the low dose groups (3 kBq ^{227}Th per mouse and 1 kBq ^{228}Th per mouse).

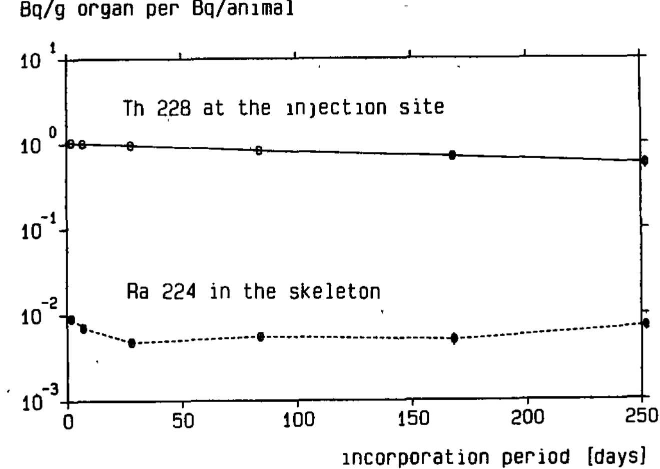


Fig. 4 Specific activity after paratibial injection of 3.1 kBq/kg ^{228}Th in colloidal form into NMRI-mice.

d) Incorporation of ^{227}Thorium at different age

37 kBq/kg ^{227}Th (corresponding to 200 cGy mean skeletal dose) were incorporated at age of 1 month or 12 months or 18 months (Fig. 5). Mice with osteosarcomas appeared in the same age range and during the age range of spontaneous osteosarcomas observed in many control experiments. This means that the latency time of the radiation-induced osteosarcoma becomes shorter with incorporation at older age. The corrected osteosarcoma incidence (calculated as long as a minimum of 10 animals were surviving) was similar for the three age groups: 32 % (1 month), 43 % (12 months), 36 % (18 months).

After incorporation of 185 kBq/kg the corrected osteosarcoma incidence was lower in the older age groups: The values were 61 % (1 month), 10 % (12 months), 15 % (18 months). Only in the group with incorporation at 12 months was an earlier occurrence of all non-bone tumours observed (data not shown).

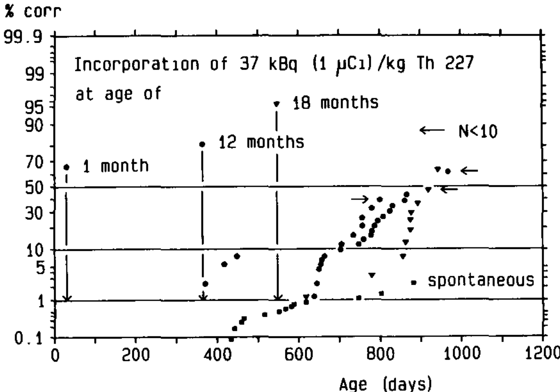


Fig. 5 Corrected osteosarcoma incidence after incorporation of ^{227}Thorium (corresponding to a mean skeletal dose of 200 cGy) at different age. Comparison with the occurrence of spontaneous osteosarcoma in 1710 untreated controls (female NMRI-mice).

e) Combination experiments

The aim was, to find possible additive or synergistic effects. In this connection the effect of injection of 1.85 kBq/kg of the long-lived radionuclide ^{227}Ac on the induction of osteosarcomas in female NMRI-mice by different dose levels (18.5, 74, and 185 kBq/kg) of the short-lived radionuclide ^{227}Th was investigated. Table II shows data and results from the experiment with young animals.

Table II Activities, doses, and final tumour incidences in the young (4-week-old) animal groups

Groups	Nuclides	Activity (kBq/kg)	No. of animals	Skeletal Dose (Gy)	Bone Sarcomas % unadjusted	Bone Sarcomas % corrected for competing risks*
C	Controls	0	50	0	0	0
A	^{227}Ac	1.85	50	1.2	12	18.1
T	^{227}Th	185	50	10.0	54	60.8
A T	^{227}Ac+^{227}Th	1.85 + 185	50	11.2	68	64.8

* value for ten surviving animals

The percentage of osteosarcoma-bearing animals after combined treatment was approximately additive, i.e., about the same as the sum of the values in the groups with ^{227}Th or ^{227}Ac alone. The *P* values of log-rank tests indicated significant or highly significant differences in times of tumour appearance between all four experimental groups.

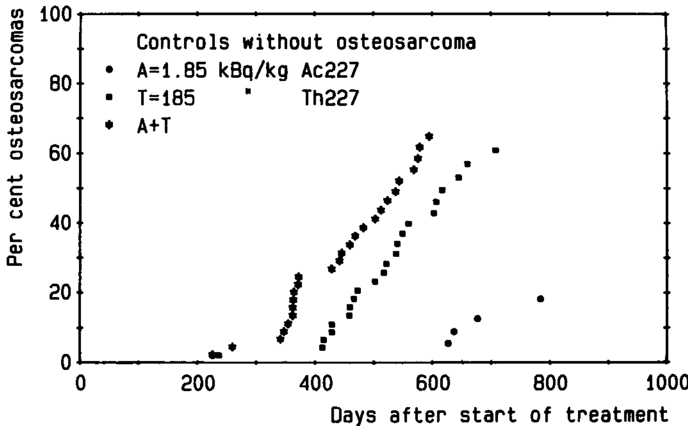


Fig. 6 Incidence of osteosarcomas in 4-week-old mice (single and combined treatments)

The cumulative incidence of osteosarcomas in the three groups is shown in Figure 6. Addition of ^{227}Ac resulted in an earlier occurrence of tumours marked by a surprisingly steep increase in osteosarcoma incidence in the period 300-400 days. This was much earlier than the occurrence of osteosarcomas in the ^{227}Ac alone group which was more than 600 days after injection (Controls = 0). Osteosarcoma incidence did not start to increase in the ^{227}Th group until after 400 days after injection, so that for the first 600 days addition of ^{227}Ac resulted in a marked increase in osteosarcoma incidence, although the final cumulative incidence in the combined and ^{227}Th alone groups was about the same. A second series of combination experiments was performed using different levels of the radionuclides as shown in detail in Table III and Fig. 7.

Table III Activities, doses, and final tumour incidences in the adult animal groups
Age of mice: 10 weeks at ^{227}Ac-injection; 12 weeks at ^{227}Th-injection

Groups	Nuclides	Activity (kBq/kg)	No. of animals	Skeletal Dose (Gy)	Bone Sarcomas % unadjusted	Bone Sarcomas % corrected for competing risks*
C	Controls	0	100	0	1	2.2
A	^{227}Ac	1.85	101	1.2	8	23.6
T1	^{227}Th	18.5	100	1.0	16	30.7
A T1	^{227}Ac+^{227}Th	1.85 + 18.5	103	2.2	30	52.0
T2	^{227}Th	74	101	4.0	47	70 6
A T2	^{227}Ac+^{227}Th	1.85 + 74	101	5.2	45	64.8
T3	^{227}Th	185	94	10.0	41	59 5
A T3	^{227}Ac+^{227}Th	1.85 + 185	104	11.2	42	63 1

* value for ten surviving animals

In the group administered 1.85 kBq/kg ^{227}Ac and 18.5 kBq/kg ^{227}Th, the time to appearance of osteosarcomas was significantly shorter ($P < 0.0260$ using the log-rank test) than in the thorium-alone group (Fig. 7). There was a higher rate of tumour appearance between 700 and 800 days in the combined group than in the other two groups added together (Fig. 7). Interestingly, the time of appearances in the thorium-alone group showed a bimodal distribution: before 650 days and after 750 days. The addition of 1.85 kBq/kg ^{227}Ac, which adds a dose of about 1.2 Gy, to the dose of 4 Gy from 74 kBq/kg ^{227}Th had no effect on either the corrected cumulative tumour incidence, the unadjusted incidence (Table III), or the tumour appearance time. In the group administered 185 kBq/kg ^{227}Th, the addition of 1.85 kBq/kg ^{227}Ac resulted in a slightly steeper but not significant increase in osteosarcoma incidence in the second half of the exposure period, but there was no effect on the final corrected cumulative osteosarcoma incidence or the unadjusted incidence (Table III).

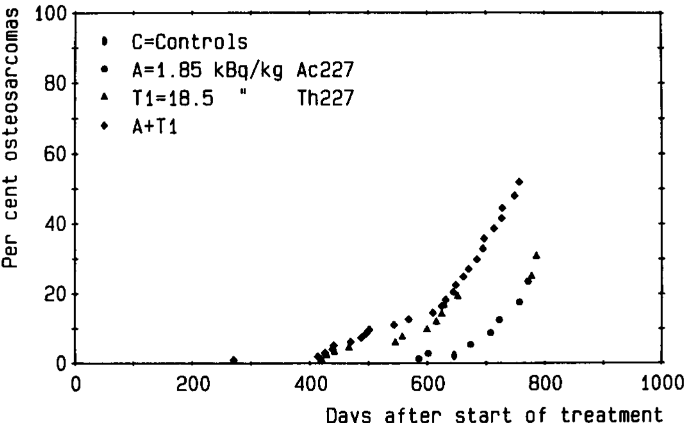


Fig. 7 Incidence of osteosarcomas in 10-12-week-old mice (single and combined treatments)

Discussion

a) The long-term experiments with fractionated internal irradiation demonstrate that the bone tumour inducing effect of the β-emitter ^{177}Lutetium can be partly abolished during an irradiation free interval. This kind of repair was not observed with the short-lived α-emitter ^{227}Thorium.

b) The most surprising result in the dose protraction experiment was the observation that protracted α-radiation at low dose levels apparently activates a different target tissue for radiation oncogenesis. Both the results reported here and those from Humphreys, Loutit, Papworth, Stones quoted in Müller et al. 1988 and 1989 show that the extrapolation of radiogenic effects towards very low doses is not only complicated with respect to the mathematical form of the dose response of a special effect (such as linear, supralinear, linear-quadratic, threshold, etc.) it also seems impossible to predict with certainty the spectrum of tumours induced.

c) The most probable explanation for the osteosarcomogenic effect of ^{144}Cerium by local application is that the high β-energy (E_{max} = 3 MeV) of ^{144}Praseodym, the decay product of ^{144}Ce, might be responsible for the local osteosarcoma induction. The local injection method of radiocolloids of thorium applied seems to be suited for radium protraction experiments, in place of repeated injection with short-lived ^{224}Radium, in long-term experiments with very low doses.

d) At a mean skeletal α-irradiation dose of 200 cGy, mice of older age (12 months, 18 months) are more susceptible to radiation-induced osteosarcoma than weanlings. At a higher level of internal irradiation dose older mice are less sensitive for radiation-induced osteosarcoma but may sometimes develop non-bone tumours earlier.

e) The results of the combination experiments show that the combined incorporation of two α-emitting radionuclides at the levels of radioactivity studied has a lower biological effect than the sum of the effects of the components administered singly. However, the effect of adding a low level of a long-lived component to a higher level of a short-lived component is variable and depends on the level of the short-lived component. Analogous results were also obtained in another combination experiment using two different doses of ^{224}Ra (18.5 kBq/kg and 185 kBq/kg) together with 1.85 kBq/kg ^{227}Ac, whereby only the combination with the lower ^{224}Ra-dose showed a significant effect of the ^{227}Ac admixture. The less-than-additive effect observed is in good agreement with experiments dealing with incorporation of a mixture of β-emitters, in which the effects are also less-than-additive (Monakhov, Shvedov quoted in Müller et al. 1990). Thus our results show that the recommendation of the ICRP, which is known to be conservative for β-emitters, is also conservative in the case of α-emitters, at least at the levels of radioactivity studied.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

V. Publications:

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Title of the project no.: 2

Pathogenesis of radiation-induced cancer

Head(s) of project:

W. Gössner

Scientific staff:

V. Erfle, J. Schmidt, P. G. Strauss, A. Schön, A. Luz, W. A. Müller, E. Schäffer, A. B. Murray

I. Objectives of the project:

The evaluation of the molecular mechanisms responsible for radiation carcinogenesis.
Investigations of exogenous factors which might modify the development and progression of radiation-induced tumours.

II. Objectives for the reporting period:

The activation of provial genes and their interaction with cellular oncogenes in radiation carcinogenesis was studied by two approaches.
First, the activation of endogenous retroviruses during the early latent period of radiation-induced osteosarcomagenesis as well as in the induced tumours, and the pathogenicity of various virus isolates was studied in different mouse strains. Radiation-induced osteosarcomas were investigated for retrovirus and protooncogene rearrangement and amplification.
Second, cell and tissue culture models were applied to establish the molecular and morphological events during normal osteogenic differentiation and to characterize retrovirus- and oncogen-induced effects on skeletal cells in vitro.

III. Progress achieved:

Radiation activation of endogenous retroviruses

Female C57Bl/6 and BALB/c mice were injected with 0.06 µCi/kg or 0.5 µCi/kg of the short-lived alpha-emitting radionuclide ^{224}Radium at 3-day intervals for two to four weeks. The continuation of this irradiation schedule results in 10% and 95% osteosarcomas, respectively. Infectious N-ecotropic XC+, and xenotropic C-type retroviruses were activated in bone marrow, spleen and bone tissue in both strains. Between 2 and 4 weeks after start of irradiation the number of C57BL/6 mice expressing infectious virus particles increased to 100%. Also the level of activated virus was up to 100-fold higher in high-dose irradiated than in low-dose irradiated mice. In BALB/c mice the levels of activated virus were within a smaller range and fewer animals responded to irradiation by virus expression.
Virus antigen concentration in the sera of irradiated and control mice were around background levels indicating the absence of viraemia at the time of investigation. In contrast, all animals showed antiviral antibodies whereby the titers in irradiated mice were higher than in controls. Taken together, these data indicate a dose-dependent activation of infectious endogenous retroviruses (OL MuLV) in the early latent period of radiation-induced osteosarcomas. High antivirus antibody titers were associated with the presence of retrovirus-specific immune complexes in the irradiated mice, which would explain the lack of free antigen in the sera of irradiated mice.

Retroviruses in neoplastic and normal bone tissue

Infectious C-type retroviruses were readily isolated from ^{227}Th or ^{224}Ra-induced osteosarcomas and from cell lines established from these tumours (OS MuLV). In the osteosarcomas of NMRI mice we found elevated concentrations of retroviral proteins with up to 1000-fold higher levels than in normal bone tissue. In parallel with these experiments we isolated C-type retroviruses from spontaneously developing benign bone tumours (OA MuLV) as well as from bone tissue of non-irradiated young adult mice (OL MuLV). From these findings we conclude, that spontaneous activation and expression of endogenous retroviruses is a common event in bone and hematopoietic tissues of different mouse strains. The process of virus activation is significantly enhanced in a dose-dependent manner by internal alpha irradiation and maintained until tumour development. Particularly high levels of viral antigens and infectious retrovirus particles were found in the irradiation-induced osteosarcomas.

Amplification and rearrangements of endogenous retroviruses and proto-oncogenes in radiation-induced osteosarcomas

BALB/c mice harbour a single copy of an endogenous ecotropic retrovirus in their genome. We have investigated the fate of this provirus in radiation-induced osteosarcomas by molecular studies of the genome of the tumours.
Extraction of DNA from small-sized primary tumours does not yield sufficient amounts for molecular analysis. We have therefore expanded primary tumour material by transplantation into syngeneic mice. DNA was extracted from these first generation transplants for further analysis. In 17 out of 29 osteosarcomas, we

found one or more newly acquired ecotropic proviruses in the genome of the tumours, and expression of viral RNA. This was in contrast to the lack of integration events in 19 clonal cell lines which were established from bone marrow cells of an irradiated, tumour-bearing mouse. Therefore, reintegration of ecotropic proviruses is a rare event in irradiated non-tumour cells, where 60% of the tumours had additional, newly acquired proviruses. New retroviral integrations with similar patterns were observed in several first generation transplants derived from the same primary tumours. From these observations we conclude, that radiation-induced osteosarcomas develop by clonal expansion of subpopulations of distinct tumour cells, and that the integration events must have occurred before transplantation.
Cellular regions flanking new proviral integration sites were molecularly cloned. Southern-blot analysis using these sequences did not detect rearrangements in any of the other tumours except in those from which they were derived. Although integration of proviruses into specific cellular regions could not be documented, the activation of ecotropic proviruses in the majority of the tumours suggest a role of these endogenous retroviruses in radiation-induced osteosarcomagenesis.

Osteogenic tumours were analysed for structure and expression of cellular oncogenes. Whereas no alterations of the gene structure were found in the regions of the genes for c-fos, c-mos or n-myc, 9 out of 30 tumours of BALB/c mice, and 3 out of 23 tumours of NMRI mice showed amplification of c-myc sequences. A region of common proviral integration in virus-induced T-cell lymphomas, Mlvi-1, localized on the same region on chromosome 15, was amplified concomittantly. In some tumours c-myc was amplified up to 40-fold. In one tumour the amplified c-myc and Mlvi-1 sequences were localized on double minutes as shown by in situ hybridization. Three tumours with c-myc amplification also showed rearrangements of the c-myc gene region. One of these rearrangements included the 5′ and 3′ flanking sequences and the noncoding part of the third exon. We found repetitive sequences in the 5′ region of the c-myc gene, and the 3′ flanking region was substituted by sequences normally present in a more distant part of chromosome 15. Increased levels of c-myc transcripts of apparently normal size were found in tumours carrying amplified c-myc sequences. Expression of c-myc was highest in the tumours harbouring approx. 40 copies of this oncogene. In these tumours the abnormally high level of steady state c-myc RNA correlated with an early stage of osteogenic differentiation, suggesting that the c-myc gene may be involved in the control of the osteogenic differentiation of neoplastic skeletal cells.

Molecular characterization of radiation-activated retroviruses

Molecular cloning of osteosarcoma and osteoma-derived viruses and comparisons with the RFB virus and Akv, the germ line-derived retrovirus of AKR mice, revealed close similarities as shown by fingerprint analysis and restriction enzyme mapping, but also distinct differences, such as rearrangements and point mutations in the enhancer-consensus region of their 3' LTR sequences. The transcriptional activity of these sequences differed also considerably as shown by chloramphenicol-acetyl-transferase (CAT) assays in osteogenic cells and fibroblast cell lines. These alterations may account for differences in the specificity of the interactions of these sequences with binding proteins, in cell-specific transcriptional activity and protein expression, and finally in the induction of aberrant differentiation processes of skeletal cells.

Pathogenicity of bone tissue-derived retroviruses

The retroviruses isolated from normal and neoplastic bone tissue represent replication competent murine leukemia viruses which do not transform mesenchymal cell in vitro. In vivo experiments, however, revealed distinct bone-pathogenic properties in susceptible newborn mice which do not carry ecotropic retroviral sequences. Besides lymphomas they induced osteopetrosis and osteomas after mean latent periods of up to 9 months in NMRI mice, whereas CBA mice developed predominantly bone lesions. All virus types tested, including the RFB osteoma virus, which was isolated by M.Finkel and coworkers from virus-induced osteomas of CF-1 mice and which was used in a series of control experiments, induced the same pattern of diseases but with great variations in incidences and latent periods.

Target cell in the skeleton and virus effects in bone cells

Newborn female NMRI mice were infected intraperitoneally with OA MuLV. Femur metaphyses and lumbar vertebrae were investigated ultrastructurally 3d, 7d, and 28d after infection. Budding, immature and mature virus was observed associated with osteoblasts and osteocytes, but never with osteoclasts or chondroclasts, 28d after infection. Skeletal tissue from infected old osteopetrotic mice showed virus particles associated with and budding from osteocytes and accumulated in devitalized osteocyte lacunae.
Primary skeletoblast cell cultures were established from humeri and calvaria of 3-day-old, 7-day-old, and 28-day-old animals in order to monitor the distribution of the virus following inoculation of newborn mice, and the degree of infection of the cells of the skeleton. Infectious ecotropic MuLV was found in all humerus cell cultures from infected mice and in 7d and 28d calvaria cell cultures. The levels of alkaline phosphatase, a marker enzyme indicative of the state of osteogenic differentiation of bone cells, were markedly higher in cultures of calvaria and humeri from infected mice than in those of from controls.
In contrast to the bone lesions found in several mutant mouse and rat strains which result from impaired osteoclasis due to dysfunctions of bone resorbing osteoclasts, the skeletal lesions induced by radiation-activated endogenous retroviruses and related virus types isolated from different bone tissues appear as a disease of the cells of the osteogenic lineage.

Effects of retroviruses in bone cell and tissue cultures

Primary cell cultures of periosteum and osteoblast-like cells were established from calvaria of newborn mice. In vitro infection of undifferentiated periosteal cells with OA MuLV was followed by a decrease in cell growth and an increase in alkaline phosphatase activity. In contrast, differentiated osteoblast-like cells were barely susceptible to virus infection, and the virus did not influence growth or differentiation of this cell population. These data indicate towards a distinct increase of osteogenic differentiation following virus infection in susceptible osteoblast precursor cells.
This phenomenon was further investigated by use of a tissue culture model, the mandibular condyle of the neonatal mouse. At the time of dissection from the mandible, this tissue is composed of distinct zones containing cartilage cells at

different stages of differentiation. Namely, mesenchyme-like cells in the perichondrium, a zone of progenitor cells in the apical and lateral part, an area of young chondroblasts in the center and mature chondrocytes in the basal part of the tissue. During in vitro cultivation over a period of 10 days, cells in the progenitor zone undergo osteogenic differentiation and form new bone. These processes are accompanied by the expression of characteristic genes typifying osteogenic differentiation including collagen type I, alkaline phosphatase, osteonectin, osteopontin and osteocalcin.

Infection of mandibular condyles with OS-5 MuLV or OA MuLV resulted in reduced growth of osteoblast progenitor cells, which have been determined by immunohistochemical methods as the target cell population of the virus in this tissue. Thereafter, the tissue showed increasing cell condensation together with polymorphism and heavy mineralization of the osteoid matrix.

Infection of mandibular condyles with the fos oncogene-bearing FBR osteosarcoma virus led to the development of an atypical proliferative osseous lesion resembling osteosarcoma, which was serially transplantable in syngeneic mice.

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RADIATION PROTECTION PROGRAMME

Final Report

Contractor:

Gesellschaft für Strahlen- und Umweltforschung mbH
Ingolstädter Landstr. 1
D-8042 Neuherberg

Contract no.: BI6-D-083-D

Head(s) of research team(s) [name(s) and address(es)]:

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Title of the research contract:

Epidemiological studies of radiation carcinogenesis and its biophysical basis.

List of projects:

1. Late effects in Ra-224 treated ankylosing spondylitis patients.
2. Late effects in Ra-224 treated juvenile and adult patients.
3. Epidemiology of radiation carcinogenesis.

*This research programme is carried out in coordination with the "Institut für Medizinische Strahlenkunde der Universität Würzburg", Prof. A.M. Kellerer and the "Kinderpoliklinik der Universität München", Prof.Dr. H. Spiess.

Title of the project no.: 1

Late effects in ^{224}Radium treated ankylosing spondylitis patients

Head(s) of project:

Prof. Dr. W. Gössner

Scientific staff:

Dr. R. R. Wick, Dr. W. A. Müller

I. Objectives of the project:

All three projects in this research programme are aimed at epidemiological studies of radiation effects in patients injected with ^{224}Radium.

Project 1 is concerned with more than 1500 ankylosing spondylitis patients treated between 1948 and 1975 with repeated intravenous injections of ^{224}Radium. The α-doses to the skeleton, on average 0.67 Gy, are considerably lower than the doses in the patients studied in Project 2. The causes of death, and occurrence of other lesions possibly related to the ^{224}Radium treatment, are analysed and compared with results in a control group of ankylosing spondylitis patients not treated with radioactive drugs or X-rays.

II. Objectives for the reporting period:

Contact and follow-up of patients of the exposure group and the control group. Registration of causes of death. Comparison of results in the exposure and control group and evaluation with respect to the risk of bone tumours, leukaemias, kidney and liver diseases, and other diseases known, or supposed from Project 2, to be related to the ^{224}Radium treatment.

III. Progress achieved:

As of December 1989, this study consists of more than 1500 ankylosing spondylitis patients (Table 1) from 9 hospitals. These patients have been collected from all orthopaedic hospitals in the FRG known to have treated notable numbers of ankylosing spondylitis patients with ^{224}Ra. The majority of these patients, most of them treated in the years 1948-1975, received one series of 10 weekly injections of about 1 MBq of ^{224}Ra each. This is the usual dosage for the present treatment of ankylosing spondylitis and leads to a cumulative α-dose of 0.56 Gy to the marrow-free skeleton of a 70 kg man.
In addition there exists a control group of ankylosing spondylitis patients not treated with radioactive drugs or X-rays in order to provide comparative information on causes of death and lesions possibly related to the basic disease itself or to chemotherapy. Patients for this control group have been drawn mainly from a hospital known to refuse ^{224}Ra treatment on principle.
Personal and treatment data for the patients of the exposure and the control group have been drawn from the hospital records. Information on the current status of patients is gained from re-examinations at different times after treatment and from questionnaires sent periodically to the patients. In course of the study or from questionnaires it has become evident that some of the patients have been treated with X-rays additionally. These patients have been deleted from the original study groups. Causes of death were determined preferably from hospital record and death certificates. For several patients also autopsy protocols were available. The underlying causes of death were classified and registered according to the 7th revision of the International Classification of Diseases (ICD).
Up till now, 500 patients in the exposure group and 636 patients in the control group have died. Causes of death have been ascertained in 487 patients of the exposure group and of 576 patients of the control group. The remaining cases are still being investigated.

Table 2 shows the skeletal and soft tissue diseases observed so far. In this table we restricted our interest to those diseases which are known or implied from Project 2, the higher dose study, to be associated with a former administration of ^{224}Ra. In the exposure group, three cases of malignant tumours in the skeleton have been observed: one fibrosarcoma, one reticulum cell sarcoma of the bone marrow, and one generalized plasmocytoma originally observed in the bone marrow of sternum and pelvis. In the control group only one case of a skeletal tumour, also a plasmocytoma, has been observed.

Diseases of the haematopoietic tissue among living and dead patients included: leukaemias (7 in the exposure group vs. 5 in the control group) and bone marrow failure (10 cases vs. 8). Also included in this table are cataracts and exostosis, two diseases which especially have been observed in the juveniles of Project 2. In the adults of this project who have been treated at considerably lower doses there is no indication of an increased incidence.
Due to the fact that for administrative reasons we follow the patients of the exposure and of the control group on different years the numbers in Table 2 are only informative. This is because we followed the control group last year, but it has been up to three years that we have followed the exposure group which will be contacted the next years. (Present follow-up time, exposure group: 16.5 yr, control group: 19.4 yr)
More exact information is given in Table 3 which shows for different kinds of cancer a comparison between observed and expected cases. The expected cases shown here have been calculated from the annual incidences of cancer for the German Democratic Republic for the years 1973 - 1977. The results obtained from other calculations with the data of the smaller West German Cancer Registries of the Saarland and of Hamburg, however, do not differ very much from the results shown here. For the calculation we grouped the patients of our exposure and control group in five year classes of age according to their individual follow-up time, that means begin of treatment until last contact or death. Table 3 shows that the number of observed total cancers for both, the exposure and the control group, is within the range of expected cases. There is also no significant difference between observed and expected cases for cancers of stomach, liver, urinary system, or the female breast. An increased incidence for stomach cancer might have been expected because of a higher intake of painkilling drugs in case of ankylosing spondylitis patients compared to a standard population. For liver, urinary systems, and female breast there are some indications from Project 2 of an excess with higher doses, which cannot be observed in our patients.

For cancers in the skeleton there might be a slight indication of an increased incidence in the exposure group (3 cases observed vs. 0.6 - 1.2 expected; p = = 0.121) whereas in the control group the observed cases are within the limitations of expected cases (1 case observed vs. 0.7 - 1.6 cases expected). The types of bone tumours in our exposure group, however, are different from those observed in Project 2: two of the three cases observed in this project were tumours of the bone marrow whereas in Project 2 mostly osteosarcomas were ob-

served. This apparent difference is somewhat parallel to the observation that in this study the leukaemias seem to be increased.

Table 4 shows a more detailed evaluation concerning the different types of leukaemias. The expected numbers for the different subtypes of leukaemia given in this table are calculated from the mortality rates in Bavaria for the years 1976 - 1981*, because an evaluation of the four-digit categories of death causes is not available from the German Cancer Registries. The numbers for expected cases given in this table are percentages as found for the different types from the Bavarian mortality rates but calculated to the basis of the expected total leukaemias (2.2, or 3.0 cases, resp.). Table 4 shows an increased incidence of total leukaemias for the exposure group, as well as for the control group (exposure group: 7 observed vs. 2.2 expected, $p = 0.0075$; control group: 5 observed vs. 3.0 expected, $p = 0.185$). Subtyping of the leukaemias reveals that in the exposure group only the chronic myeloid leukaemias (3 cases vs. 0.7 expected, $p = 0.034$) are significantly elevated whereas in the control group the acute lymphoblastic leukaemias (3 cases vs. 0.2 expected, $p = 0.0011$) are increased.

For the control group this might be due to a higher intake of painkilling drugs compared to the patients exposed to ^{224}Ra. It is well known that phenylbutazone, a drug which was widely used in the treatment of ankylosing spondylitis, can cause bone marrow depression. Acute leukaemias occurring in association with phenylbutazone treatment have repeatedly been reported in the literature.

Whereas an increase of the rate of the tumours in the skeleton is uncertain, we observed in the exposure group more chronic myeloid leukaemias than expected. As the average α-dose to the skeleton in the patients of our study group is lower by a factor of about 3 than in the adults and lower by a factor of about 17 than in the juveniles of Project 2, effects on the bone marrow not observed there may be prevalent. Certain disorders of the haematopoietic system following treatment with ^{224}Ra were observed even at the same low dose level by other authors.

Myeloproliferative diseases, mostly acute or chronic myeloid leukaemias, have been reported following Thorotrast applications too (see Table 5): 10 cases were observed among 432 deaths in a group of 878 well examined Thorotrast patients followed in the Federal Republic of Germany. Also included in this table are three cases of myeloid leukaemia in the adult group of Project 2. No case of myeloid

* We would like to thank B. Grosche from the Bundesgesundheitsamt, Institut für Strahlenhygiene, Neuherberg for providing us with these data.

leukaemia is reported in Project 2 for the juveniles who received the highest doses. From Table 5 a correlation of the bone marrow *dose* with the incidence of myeloproliferative diseases cannot be demonstrated, an effect of the *dose-rate*, however, may be taken into consideration (Fig. 1). The relatively high incidence of myeloid leukaemias in the Thorotrast patients seems very surprising because of the very low dose rate of only 1.7 mGy per week of bone marrow dose. On the other hand, higher total doses, but also at higher dose rates produced no myeloid leukaemias in the juveniles of Project 2. The dose rates, as well as the incidences of myeloid leukaemia, for both, the patients of this project and for the adults of Project 2, lie in between.

Although the number of cases is low for the groups at the higher dose rates one should consider that not only the total dose but also the dose rate may play an important role in the induction of malignancies in man. In animals, bone seeking α-emitters given at very low dose rates, lower than those found to cause bone tumours, have been demonstrated by different authors to induce leukaemias. Results from an experiment with varying amount of ^{224}Ra performed by *Humphreys et al.* (1985) indicate that animals given amounts of ^{224}Ra less than those found to cause osteosarcomas may be at risk instead from myeloid leukaemia. Also for ^{239}Plutonium, a bone surface seeker like ^{224}Ra, the induction of myeloid leukaemias has been demonstrated in mice down to dose rates of a few mGy/day or even less. In our institute (CEC-Contract No. BI6-D-080-D) α-doses to the marrow as much as one order of magnitude lower (30 - 200 mGy) than those reported up to now were found to be leukaemogenic when delivered over a long period at an extremely low dose rate. The types of these leukaemias, however, were different to those reported by other authors.

A hypothesis on cellular mechanisms for the induction of leukaemias at low dose rates has been set up by *Thorne* and *Vennart* in 1976. They postulated an intermediate state of cells transformed by radiation which is radiation sensitive itself. This results in an equilibrum level of cells transformed or sterilized by radiation. At higher doses the sterilisation of transformed cells exceeds their production and less or no leukaemias are observed in the irradiated animals. Only at low dose rates the radiation-induced leukaemias can be observed. This hypothesis might fit for the induction of leukaemias not only for animals but also for the observations made for humans in this project.

Table 1: ANKYLOSING SPONDYLITIS PATIENTS IN THE EXPOSURE AND CONTROL GROUPS (Dec. 1989)

	Exposure Group	Control Group
Total patients	1579	1461
Treated with X-rays additionally	106	126
Remaining patients	1473	1335
Deceased patients	500	636
Cause of death certified	487	576
Cause of death not yet known, still in work	13	60

Table 2: SKELETAL AND SOFT TISSUE DISEASES (Dec. 1989)

	Exposure Group	Control Group
Observed patients	1473	1335
Deceased	487	576
Total cancers	80 (+14*)	116 (+12)
Skeleton	3	1
Leukaemias	6 (+ 1)	5
Acute leukaemia	1	4
Chronic leukaemia	5	1
Type unspecified	0 (+ 1)	0
Bone marrow failure	4 (+ 6)	5 (+ 3)
Cataracts	3 (+25)	1 (+16)
Exostosis	0 (+ 1)	1

* living

Table 3: CANCERS OF BONE AND SOFT TISSUE
(Incidence *obs/exp* as of Dec. 1989)

	Exposure Group		Control Group	
	obs	*exp*	*obs*	*exp*
Total cancers	94	97.7	128	139.8
Stomach	14	11.1	15	17.0
Liver	1	2.9	5	4.3
Urinary System	6	6.4	8	9.6
Female Breast	1	2.0	1	1.7
Skeleton	3	0.6-1.2	1	0.7-1.6
Leukaemia	7	2.2	5	3.0

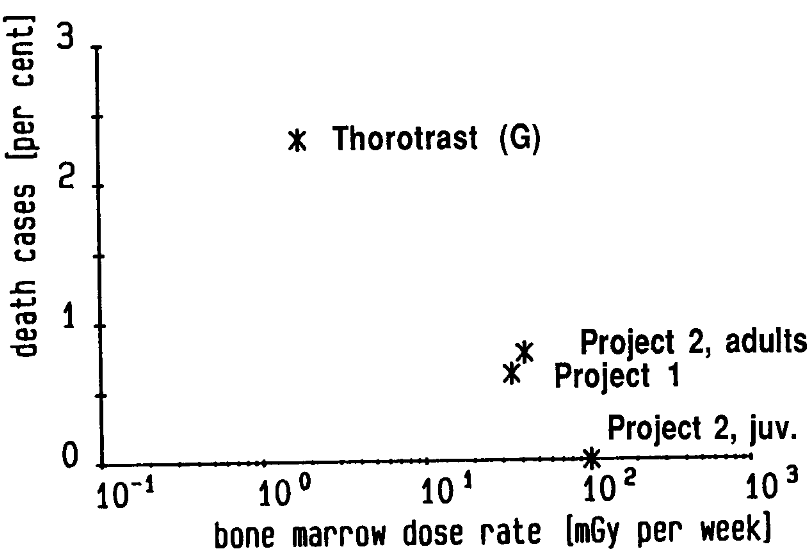
Table 4: LEUKAEMIAS IN ^{224}Ra TREATED ANKYLOSING SPONDYLITIS PATIENTS (Dec. 1989)

	Exposure Group		Control Group	
	obs	*exp*	*obs*	*exp*
Total leukaemias	7	2.2	5	3.0
Acute leukaemias	1	(0.7)	4	(0.9)
Acute lymphoblastic leukaemia	1	(0.2)	3	(0.2)
Acute myeloid leukaemia	0	(0.5)	1	(0.7)
Chronic leukaemias	5	(1.5)	1	(2.1)
Chronic lymphatic leukaemia	2	(0.8)	0	(1.1)
Chronic myeloid leukaemia	3	(0.7)	1	(1.0)
(Type unspecified	1		0	)

Table 5: MYELOPROLIFERATIVE DISEASES IN PATIENTS TREATED WITH ^{224}Ra OR THOROTRAST (Dec. 1989)

	Total pat.	Deceased patients	Marrow dose <Gy>	Burden time <week>	Dose per week <mGy>	Myeloproliferative Diseases	
						# cases	per cent
Project 2 Juveniles	218	90	5.3	51	104	0	0
Project 2 Adults	682	439	1.0	25	41	3	0.68
Project 1	1473	487	0.34	10	34	3	0.62
Thorotrast Study (Examined patients)	878	432	3.0	1768 (34 yr)	1.7	10	2.91

Figure 1: DEATH CASES FROM MYELOPROLIFERATIVE DISEASES



IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

10 Orthopaedic and Rheumatic Hospitals from the F.R. Germany and West Berlin.

Close cooperation with the working groups in Projects 2 and 3 of this contract.

V. Publications:

1. Wick, R. R., Chmelevsky, D., Gössner, W.:
^{224}Ra: Risk to bone and haematopoietic tissue in ankylosing spondylitis patients.
Strahlenther. **Suppl. 80**, 38-44 (1986)

2. Wick, R. R., Spiess, H., Gössner, W.:
Long term effects after therapeutic administration of different doses of ^{224}Ra (Thorium X).
Intern. J. Radiat. Biol. **49**, 876 (1986)

3. Wick, R. R., Chmelevsky, D.:
Risiko somatischer Strahlenspätschäden des Menschen nach Inkorporation von ^{224}Ra (Thorium X).
GSF-Bericht **8/86** (1986)

4. Rabenseifner, L., Wick, R. R.:
Spätergebnisse nach Radium-224 Behandlung bei ankylosierender Spondylitis.
Akt. Rheumatol. **11**, 223-226 (1986)

5. Gössner, W.:
Pathology of radiation-induced bone tumors.
Leuk. Res. **10**, 897-904 (1986)

6. Wick, R. R.:
Ergebnisse einer Follow-up Studie ^{224}Ra-behandelter Bechterew Patienten.
Akt. Rheumatol. **12**, 33-37 (1987)

7. Wick, R. R., Gössner, W.:
^{224}Ra in man: Long term effects on bone and haematopoietic tissue.
Eighth International Congress of Radiation Research, Edinburgh (UK), July 19-24, 1987, D43-8V, Book of Abstracts, p. 210

8. Luz, A., Müller, W. A., Linzner, U., Murray, A. B., Wick, R. R., Gössner, W.:
Die Zeit als Kofaktor des Strahleninduzierten Knochentumors.
In: Fortschritte der Osteologie in Diagnostik und Therapie (Hrsg.: F.H.W. Heuck, E. Keck), Berlin: Springer-Verlag, S. 119-124 (1988)

9. R. R. Wick, W. Gössner:
Recent results of the follow-up of radium-224-treated ankylosing spondylitis patients.
In: BIR Report No. 21, Risks from Radium and Thorotrast (Eds.: D. M. Taylor, C. W. Mays, G. B. Gerber, R. G. Thomas). London: British Institute of Radiology, 25-28 (1989)

10. R. R. Wick:
Late effects in humans after intravenous treatment with Radium-224.
EULEP Newsletter No. **52**, 6 (1989)

Title of the project no.: 2

Late effects in ^{224}Radium treated juvenile and adults patients

Head(s) of project:

Prof. Dr. H. Spiess

Scientific staff:

Prof. Dr. M. Jensen, J. P. Scharff

I. Objectives of the project:

Follow-up initiated in 1948 of patients treated with ^{224}Ra as juveniles or adults.

Determination of stochastic and non-stochastic radiation effects and their dose, time, and age dependence.

II. Objectives for the reporting period:

Cooperation with Prof. F. H. Stefani (Eye Hospital of the University of Munich).

Cooperation with Prof. C. W. Mays (National Cancer Institute/NIH, Radiation Epidemiology Branch, Bethesda/MD, USA) in the cataract and kidney study.

Review of existing data.

III. Progress achieved:

1. Methodology

At 3 year intervals we are following the health of 900 Patients (509 men, 173 women, 111 boys, and 107 girls) who received repeated injections of ^{224}Ra after World War II, for treatment of ankylosing spondylitis or bone tuberculosis, but also for other non-cancerous diseases.

On the average, the dosage in kBq/kg was about twice as high to the children as to the adults, and because of the growing skeleton, the average skeletal dose was much higher in the children than in the adults. Usually the patients received 1 or 2 injections per week. The time spans were averaging 6 months for the adults and 11 months for the juveniles. In our 1987-1988 survey the patients' follow-up times ranged from 0 to 43 years, averaging 24 years. At the time of last contact 529 of 900 patients were deceased.

The short, 3.62 days half-life of ^{224}Ra causes some of its decay in the skeleton to take place on bone surfaces and some within bone volume, giving a local distribution of α-particle dose similar to that from ^{239}Pu. The calculated doses to the patients' skeletons (without marrow) ranged from 0.06 to 57.5 Gy, averaging 4.16 Gy. Based on results in beagles (Lloyd et al., 1982) the average injection dosage of 666 kBq/kg body mass to these patients delivered an average α-particle dose of about 1.4 Gy to the liver and 2.3 Gy to the kidneys. We estimate very crudely the α-particle dose to the red marrow to average 2 Gy. However, this dose is non uniform, being highest near the bone surface and lowest in the central marrow. Unfortunately, no estimates of the dose to the glandular breast tissue are available: experimental evaluation of the breast dose from ^{224}Ra and progeny in suitable experimental animals is urgently needed to interpret properly the observed excess of breast cancers among the juvenile girls injected with ^{224}Ra.

2. Results

Bone sarcomas

These have occurred in 54 patients, with two developing a second primary sarcoma, for a total of 56 bone sarcomas (compared to 0.2-0.3 cases expected naturally). Thus we conclude that virtually all of these bone sarcomas were radiation--induced. The dose response has been fitted to a linear-quadratic-exponential equation and the wave-shaped distribution of tumour appearence times has been analysed. The earliest bone sarcoma appeared 3.5 years after the start of ^{224}Ra injections. The incidence rate peaked at 8 years and then declined. The last of

such malignancies appeared in 1983, 33 years after injection. At low doses of ^{224}Ra, a cumulative lifetime risk from bone sarcoma of about 0.8 % per Gy of skeletal dose is inferred from our data that pertain mainly to high doses. The observation of only three tumours of the skeletal region among 1473 low-dose patients averaging 16.5 years follow-up and 0.65 Gy to the skeleton suggests that the risk coefficient at low doses may be even less.

Soft-tissue malignancies

There were 102 observed soft tissue malignancies, and this number is close to the expectation for the age distribution and person-years of observation, with the age-specific incidence rates from the Cancer Registry of Hamburg, West Germany, or the Cancer Registry of the German Democratic Republic, respectively. The close agreement between the observed and expected rates suggests that, most of the soft-tissue malignancies were not induced by ^{224}Ra but were due to other factors, but certain types of cancer were elevated in incidence.

Breast cancer

The breast cancer excess came as a surprise with 15 cases observed versus 4.1-6.1 cases expected. All but one case occurred more than 10 years after irradiation and all but one occurred after the age of 35. In the patients given ^{224}Ra as adults the six cases observed are statistically still consistent with the 3.5-5.2 expected cases. However, for those given ^{224}Ra as juveniles, a tenfold increase occurred (nine observed versus 0.6-0.9 expected, $p << 0.001$). Of especial interest are two cases that received ^{224}Ra before the age of 10, which was prior to the proliferation of breast tissue during puberty. In the A-bomb survivors, for comparison, the risk from radiation-induced breast cancers was higher in those irradiated at the age of 0-9 and 10-19 years, than in those irradiated as adults (Tokunaga et al., 1987). External X-rays can increase the risk of breast cancer, but no evidence was found in the patients' records of any X-ray exposure before the diagnosis of breast cancer. The four living breast cancer patients were interviewed, and they reported no radiotherapy and no lung collapse therapy accompanied by fluoroscopy. In three of our dead patients with breast cancer who received ^{224}Ra as juveniles, pneumothorax was unlikely because they were treated by Dr. Paul Troch who did not use pneumothorax. He believed in ^{224}Ra above any alternative therapy. In the remaining "juvenile" case, pneumothorax was possible but not considered very likely, especially after receiving the antituberculosis drug isoniazid (INH) in 1953 for pulmonary tuberculosis. Thus, even if a few of the nine juvenile cases received

pneumothorax it could not account for their observed relative risk of ten (9 cases observed vs. 0.9 expected).
Women weighing over 70 kg had a relative risk of about 1.2 for breast cancer, compared to women of all adult weights in the study of Brinton et al. (1979). With known adult weights and heights for eight of nine juveniles with breast cancer and photographs including the remaining case, we classify two as obese, six as normal and one as thin. Thus, the effect of their weight on the risk from breast cancer seems minor.
The nine juvenile breast cancer patients received the ^{224}Ra injections during the years 1943-1950 when drugs against tuberculosis were not widely available in Germany. In general the tuberculosis patients took relatively few drugs, in contrast to the ankylosing spondylitis patients who took large amounts of drugs over prolonged times to suppress pain. Thus, we have no evidence that the nine cases of breast cancer might have been drug induced. Women with either a mother or a sister with breast cancer have a relative risk of about two for breast cancer compared to other women (Brinton et al., 1982). In our interviews four patients with breast cancer reported cases of second degree relatives with breast cancer. Four cases who died before could not be interviewed.
Whatever other factors may be responsible for an excess of the breast cancer rate, it seems unlikely to be drugs, obesity, delayed childbirth, external irradiation, or the original disease of tuberculosis. A possible role of the ^{224}Ra and its decay products, can thus not be excluded and is in line with the extensive data that show that ionizing radiation can induce breast cancer (Shore, 1986; Tokunaga et al., 1987; Hrubec et al., 1989).
The average year of breast cancer diagnosis was 1977, and as of that time the 62 surviving women who were treated as juveniles had an average dose of 740 kBq per kg, a value similar to the 800 kBq/kg among the seven patients of known dosage with breast cancer. If breast cancer had occurred predominantly at the higher dosages it would indicate more strongly the possibility of breast cancer induction by ^{224}Ra.

Liver cancer

These have ocurred in six ^{224}Ra patients, i. e. in a significantly larger number than the 1.1-1.2 expected cases ($p < 0.002$). One "probable" liver cancer might possibly have been a metastasis, but the clinical and pathology reports indicate clearly that the remaining five were primary liver cancers, and not metastasis from the gallbladder or external bile duct. Even for the five confirmed cases versus

1.1-1.2 expected, the difference is still significant ($p < 0.008$). Three of the liver cancers were bile duct carcinomas, the most frequent form of liver cancer observed following α-particle irradiation of the 2334 German Thorotrast patients in which 347 liver cancers had been recorded as of the year 1984 (Van Kaick et al., 1986). A possible complication is that two patients had hepatitis of unspecified form and another one had hepatitis B. Hepatitis B increases the risk for hepatic cell carcinoma, but has not been associated with an increased risk for the bile duct carcinoma, and the latter occurred in the patient who had hepatitis B. None of the patients' records indicated an intake of drugs that might be liver carcinogens or an occupational exposure to solvents, arsenic or vinylchloride. Two of the patients with liver cancer told us that they rarely drank alcohol. Using the dosimetry of ^{224}Ra in the beagle liver (Lloyd et al., 1982) the calculated liver doses to the six patients with liver cancer average 1.9 Gy, compared to 1.4 Gy for all of the patients of known dosage in our series. Based on our results and the Thorotrast experience, we feel that most of the six liver cancers were radiation-induced and that more will come in the future. Perhaps a reason that more have not appeared already is that the span of injections in the patients with liver cancer ranged from 3 to 27 months, averaging 11 months. Thus the period of irradiaton from the short-lived ^{224}Ra was brief and did not provide the continuous killing of liver cells and promotion of cell division.

Kidney cancers

Land (1986) has reviewed the susceptibility of the kidney to radiogenic cancer. In our study, these have occurred in six patients versus 2.4-2.6 expected cases ($p < 0.05$). All of these cancers were "hypernephromas". The tumour appearence times ranged from 12 to 35 years after the start of ^{224}Ra injections.

The six cases with kidney cancers were not associated with use of drugs known to cause kidney damage, nor with occupational exposure to solvents, cadmium or mercury. Using the dosimetry of ^{224}Ra in the beagle kidney (Lloyd et al., 1982), the calculated kidney dose to the five patients with kidney cancers averaged 3.5 Gy, compared to 2.3 Gy for all the patients with known dosage in our series. Non-malignant diseases of the kidney were reported for 80 patients in our series of whom 60 have died, mainly from kidney disease. In contrast, only about four deaths from kidney disease should have been expected based on German mortality statistics. We aim at a detailed analysis of the non-malignant kidney diseases in the future.

Leukaemia

Leukaemias occurred in six patients, compared to two cases expected according to German Cancer Statistics. However some of the ankylosing spondylitis patients took leukaemogenic drugs, such as phenylbutazone to suppress pain. The elevated leukaemia rates observed in the control group of Project 1 predict five leukaemia cases unrelated to radiation among the ^{224}Ra spondylitis patients, while the German population rates predict 2.2 leukaemia cases among the ^{224}Ra patients.

Indeed, of the six leukaemias among the ^{224}Ra patients of our study, four occurred among the 396 spondylitis patients, but only two among the 504 other patients who mostly had tuberculosis for which few drugs were available at that time in Germany. Only one of the juvenile patients has developed leukaemia and she, a tuberculosis patient, had chronic lymphatic leukaemia, a type not associated with radiation exposure. For all the patients in our series, the α-particle dose to red marrow is crudely estimated to average roughly 2 Gy. No excess of leukaemia has been observed in the US or British radium dial painters (Spiers et al., 1983; Baverstock & Papworth, 1986).

Other cancers

These have not yet shown a significant excess in the ^{224}Ra patients, including stomach (11 versus eight to ten expected), lung (16 observed versus 18-20 expected), skin (four observed versus 2-11 expected), uterus (five observed versus 3-5 expected), ovary (two observed versus one to two expected), prostate (six observed versus five to seven expected), bladder (four observed versus three to four expected), brain (two observed versus one to two expected), and multiple myeloma (two observed versus 0.5-0.6 expected). One of each of the following types occurred: Hodgkin's lymphoma, cancer of parotid gland, pancreas, thyroid, oesophagus, and fallopian tube. Six patients had cancers of unknown type.

Two negative findings are of special interest. First, no melanomas were observed in the ^{224}Ra patients, although melanomas have been induced in the eyes of beagles injected with radium isotopes (Taylor et al., 1972). However, it is possible that radium concentrates more in dog melanocytes (pigmented cells) than in human melanocytes. Second, no carcinomas of the head sinuses were observed in the ^{224}Ra patients, although 37 head sinus carcinomas have occurred in US persons internally contaminated with ^{226}Ra (Gustafson & Stehney, 1985). The ^{226}Ra decays to 3.8 day ^{222}Rn gas which diffuses into the head sinuses and irradiates the squamous epithelium. In contrast, as ^{224}Ra decays to ^{220}Rn gas (thoron, 56s half-life), its short half-life limits its diffusion into the head sinuses.

Statistical and radiobiological evidence suggests that ^{224}Ra and its decay products can induce bone sarcomas and may induce breast cancers, liver, and kidney cancers in humans. In the continued follow-up, the possibility of increased rates of additional types of cancers will need to be monitored.

Cataracts

See radiation protection programme, progress report 1989.
(Contract BI6-D-221-D)

Table 1: SUMMARY OF THE ^{224}Ra PATIENTS IN PROJECT 2 (Dec. 1989)

Patient No.	Category	Alive	Dead	Total	ThX
1 - 368	Men, Ankyl. spondyl.	122	246	368	114
401 - 516	Men, Tb	45	71	116	1
601 - 625	Men, other	5	20	25	3
	Total men	172	337	509	118
701 - 724	Women, Ankyl. spondyl.	10	14	24	8
801 - 924	Women, Tb	55	69	124	0
951 - 975	Women, other	6	19	25	8
	Total women	71	102	173	16
	Total adults	243	439	682	134
1101 - 1117	Boys, 1- 5 y	11	6	17	0
1201 - 1235	Boys, 6-10 y	21	14	35	0
1301 - 1328	Boys, 11-15 y	19	9	28	0
1401 - 1431	Boys, 16-20 y	16	15	31	1
	Total boys	67	44	111	1
1501 - 1517	Girls, 1- 5 y	7	10	17	0
1601 - 1635	Girls, 6-10 y	22	13	35	0
1701 - 1725	Girls, 11-15 y	17	8	25	0
1801 - 1830	Girls, 16-20 y	15	15	30	0
	Total girls	61	46	107	0
	Total juveniles	128	90	218	1
	Total patients	**371**	**529**	**900**	**135**

Table 2: SUMMARY OF DISEASES OF THE ^{224}Ra PATIENTS IN PROJECT 2 (Dec. 1989)

	age at first injection		
	1-20 yr	adult	total
Skeletal diseases			
Bone sarcoma	38	18	56
Exostosis	29	0	29
Growth retardation	28	0	28
Tooth breakage	40	20	60
Soft tissue diseases			
Cataract	38	47	85
Leukaemia	1	5	6
Liver (non-cancer)	4	35	39
Kidney (non-cancer)	11	69	80
Diabetes	3	28	31
Cancers of soft tissue			
Lung	1	13	14
Female Breast	9	6	15
Uro-genital region	1	17	18
Stomach	2	17	19
Skin	1	3	4
Liver	1	5	6
Kidney	1	5	6
Brain	2	8	10

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

- Prof. Dr. C. W. Mays, National Cancer Institute/NIH, Radiation Epidemiology Branch, Bethesda/MD, USA; deceased in 1989

- Prof. Dr. F. H. Stefani, Eye Hospital, LM University of Munich

- Prof. Dr. Gurland, Klinikum Großhadern, LM University of Munich

- Prof. Dr. E. Sonnabend, Dental Clinique, LM University of Munich

- Dr. D. Chmelevsky, Gesellschaft für Strahlen- und Umweltforschung, Institut für Strahlenschutz, Neuherberg

V. Publications:

1. Mays, C. W., Rowland, R. E., Stehney, A. F.:
   Cancer risk from the lifetime intake of Ra and U isotopes.
   Health Phys. **48**, 635-647 (1985)

2. Mays, C. W., Spiess, H., Chmelevsky, D., Kellerer, A.:
   Bone sarcoma cumulative tumor rates in patients injected with ^{224}Ra.
   Strahlenther. **Suppl. 80**, 27-31 (1986)

3. Chmelevsky, D., Kellerer, A. M., Spiess, H., Mays, C. W:
   A proportional hazards analysis of bone sarcoma rates in German 224-Radium patients.
   Strahlenther. **Suppl. 80**, 32-37 (1986)

4. Spiess, H., Mays, C. W., Spiess-Paulus, E.:
   Growth retardation in children injected with ^{224}Ra.
   Strahlenther. **Suppl. 80**, 45-50 (1986)

5. Stefani, F. H., Spiess, H., Mays, C. W.:
   Cataracts in patients injected with ^{224}Ra.
   Strahlenther. **Suppl. 80**, 51-59 (1986)

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   Radiat. Environ. Biophys. **27**, 103-114 (1988)

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Health Phys. **56** (Suppl. 1), 45 (1989)

11. Mays, C. W.:
Wie gefährlich ist Plutonium?
In: Plutonium, GSF Magazin "Mensch und Umwelt", 6. Ausgabe, Neuherberg: Gesellschaft für Strahlen- und Umweltforschung, 45-50 (September 1989)

Title of the project no.: 3

Epidemiology of radiation carcinogenesis

Head(s) of project:

Prof. Dr. A. M. Kellerer

Scientific staff:

Dr. J. Breckow, M. Depner, H. Friede

I. Objectives of the project:

The project is aimed at the further development of mathematical methods for the analysis of the dose, age, and time dependences of radiation-induced neoplasms. It is equally concerned with the application of these methods to animal studies and to epidemiological investigations. Risk estimates for low doses of ionizing radiations need to be based on a synopsis of essential results obtained from animal studies and from the major human epidemiological investigations. Such a synopsis requires the utilization of comparable mathematical methods and models; the efforts in this project were focussed on this need. The joint analysis of dose, age, and time dependences is particularly important in view of the new risk estimates being obtained after the revision on the Japanese dosimetry and also in view of the increased interest, after the reactor accident in Chernobyl, in the problem of low dose of ionizing radiations. The great public concern about the risks even of small doses have given added urgency to this work.

II. Objectives for the reporting period:

This project was executed in close coordination and collaboration with Projects 1 and 2 within this research program. It had two major and equally important aims. The first aim has been the statistical and mathematical evaluation of the data from the ^{224}Radium follow-up studies. This work has been mainly directed towards the analysis of the dose, time, and dose-rate dependences of the bone sarcomas in the ^{224}Radium patients, and the analysis of analogous interrelations for the increased occurrence of lens opacifications.
The second major purpose of the project was methodological work aimed at the improvement of the mathematical methods that are equally applicable to animal studies and epidemiological investigations of radiation carcinogenesis. This work included also the analysis of the extended observations among the atomic bomb survivors after the revision of the atomic bomb dosimetry, joint work with Czechoslovakian colleagues on the evaluation of lung cancer data in uranium miners, and quantitative evaluation of lung cancer data obtained in animal studies by the group of Lafuma and colleagues in Fontenay-aux-Roses.

III. Progress achieved:

Studies accompanying the follow-up of the ^{224}Radium patients

Spiess, Mays, and Stefani had earlier in their follow-up of the ^{224}Ra patients recognized an increased frequency of lens opacifications; in the few cases with detailed ophthalmological investigations they were able to see typical characteristics of radiation-induced cataracts. A detailed retrospective investigation of the degree of cataracts among all patients is impossible, but it appeared more and more important to preserve and extract information on lenticular damage gained during the follow-up of the ^{224}Ra patients. A thorough mathematical investigation of all diagnoses of cataracts and of their dependence on injected activity, age at treatment, age at diagnosis, and certain other factors was therefore performed. The data were restricted to information on loss of visual capacity, ascertained by the ophthalmologists' reports, which were obtained by slit-lamp investigations.
The mathematical investigations were critically dependent on the possibility to establish reliable dependences on dosage of ^{224}Ra, age at treatment, and time to the recognition of visual impairment. A mere correlation between the ^{224}Ra injections and the enhanced frequency of cataracts would not have been sufficient to establish a causal relation between the α-ray exposure and the lenticular damage; the possibility of enhanced frequencies due to the original illness (specially bone tuberculoses) could not be excluded by such simple analysis.
A stepwise procedure of non-parametric and parametric evaluation of the data was, therefore, utilized to obtain dependences on dosage for the different ages at treatment and diagnosis. The first level of the investigation was the construction of suitable scatter diagrams to permit a direct visual judgement of the data. The second step of the analysis was a non-parametric, i. e. model independent, maximum likelihood analysis. The final step was an evaluation in terms of suitable analytical functions in time and in dosage.
The analysis has demonstrated that almost all of the "early cataracts", i. e. those appearing before an age of roughly 53 years, were due to the ^{224}Ra injections. In line with the postulate of a non-stochastic radiation effect, i. e. radiation action on a multiplicity of cells, the dosage dependence was found to be consistent with a threshold of about 0.5 MBq ^{224}Ra per kg bodyweight, or with a quadratic dependence on dosage. A second notable finding was the virtual independence of the frequency of radiation-induced cataracts on age at treatment; the more evident induction of radiation-induced opacities in the younger patients was merely due to

the fact that the radiation-induced opacities were clearly separated from the later spontaneous age dependent cataracts.
The result of these studies suggested the urgent need, to salvage the remaining information on subclinical lenticular damage and its evolution in this singular collective of patients. A new research project was, therefore, initiated in which the surviving patients are regularly called in for specific ophthalmological investigations.
The most severe late effect of the ^{224}Ra injections was the induction of more than 50 bone sarcomas among the 900 ^{224}Ra patients of Project 2. This data set permits, perhaps more than any other epidemiological observations, the precise determination of dose and time dependences of radiation-induced cancers. The statistical studies were, therefore, extended to include additional aspects. The application of a proportional hazards analysis that was non-parametric in time but linear-quadratic in mean skeletal dose led to risk estimates which were less by about a factor of two than earlier values. The non-parametric analysis was later followed by a parametric evaluation that utilized a log-normal model for the time dependence, establishing the connection to a formalism utilized in the radioepidemiological tables of NIH. This parametric analysis provided risk estimates which were consistent with those obtained in the non-parametric approach.
A major effort in the project was aimed at the assessment of a possible dose-rate dependence for the ^{224}Ra induced bone sarcomas. An increased probability for bone sarcomas at specified injected activities but longer protraction times had earlier been surmised by Spiess and Mays, but there was a need to base the conclusions on a rigorous mathematical evaluation. To avoid bias by assumed models, non-parametric test procedures were developed which permitted the assessment of a dose-rate dependence with no assumptions on the shape of the dose dependence and with full account for the time at risk of each patient. The result confirmed the existence of the inverse dose-rate effect. A non-parametric maximum likelihood fit was then employed, to identify the underlying dose and dose-rate dependence quantitatively. This led to the remarkable finding that one obtains linear dose dependences for the ^{224}Ra induced bone sarcomas, if different doses, i.e. different injected activities, are given within a constant time. In contrast one obtains linear-quadratic dependences, when the dose rate is constant, i. e. when higher doses are given within proportionally extended time periods. The dependence on dose rate is not a small factor; when the same activity is administered during a period of 1 year rather than in 2 months the osteosarcoma frequency is increased by a factor of 2. The result is in line with a growing number of findings of inverse dose-rate effects for densely ionizing radiations. In this study

there is no indication that the reversed dose-rate effect is more expressed at the higher than at smaller doses. However, it must be kept in mind that even the smallest doses of about 0.1 MBq per kg bodyweight are still large compared to doses directly relevant to radiation protection.
The application of the results from the follow-up by Spiess et al. is, however, of immediate interest with regard to the concurrent epidemiological investigation of the more recent low dose ^{224}Ra patients which is the objective of Project 1. The results of both studies are at present consistent, however the low dose study is still incomplete. A sharper comparison of the results is expected in several years. The most recent findings in Project 2 concern an enhanced frequency of other neoplasms such as mammary carcinomas. Unlike the bone sarcomas these findings are subject to uncertainties with regard to the spontaneous incidence of the tumours, and this has led to initial work in this project on the establishment of age specific cancer rates within the patients of the Spiess series.

Methodological work and statistical evaluation of other studies

The statistical evaluation of the ^{224}Ra follow-up studies necessitated the further development of numerical techniques and of mathematical models for the determination of dose and time dependences. These mathematical aspects of the research project were, however, treated in a broader context and were aimed at the development of general tools applicable to epidemiological studies of radiation cancerogenesis and animal studies.
There were three main aspects. The first aspect concerned the improvement of maximum likelihood methods for model fits to data sets that are dependent on a variety of factors. For this purpose the flexible and highly efficient non-linear optimization algorithm of *J. Abadie* was adapted to the use on small computers. Simpler maximum likelihood algorithms, mostly based on the Newton-Raphson method, were sufficient for other purposes. The range of different algorithms will be used as a basis to develop a set of multipurpose programs for radiation studies.
The numerical evaluation of complex data sets is of necessity abstract, and it is therefore difficult to judge the validity of the approach and the degree to which a model and its parameters are defined by the underlying data. To remove some of these difficulties and to permit a direct judgement of the data, broad use was made of graphic procedures and especially of scatter diagrams of the input data. From a mathematical standpoint such diagrams are simple, however, they have proved highly useful in a variety of applications, and they will be further extended.

A third component of the mathematical investigations is the development of improved non-parametric tests for right censored and double censored data. The need and the use of such tests is most clearly exemplified in the study on the reversed dose-rate effect for the induction of osteosarcomas.
The mathematical methods have been employed in the ^{224}Ra follow-up studies, but they have also been utilized for generating a synopsis of the cancer mortality data from Hiroshima and Nagasaki. The utilization of the numerical procedures in terms of the proportional hazards analysis will have to await the release of the most recent data based on the revised dosimetry.
Extensive studies have been performed in the continued collaboration with the group of Lafuma and colleagues at Fontenay-aux-Roses on the induction of lung cancers in Sprague Dawley rats by radon daughter inhalations, neutron exposures, and gamma-irradiations. The high RBE values of radon daughter exposures and of neutron exposures in comparison to gamma rays were confirmed in this study. The analysis has furthermore provided evidence for a higher relative efficiency of radon inhalations compared to α-rays than the one that would correspond to quality factors which are currently employed.
A recent effort in the project was directed towards a cooperation with Czechoslovakian colleagues on the evaluation of lung cancer incidence in uranium miners. Their important data sets have not been part of the joint analysis of occupational radon inhalation data in the BEIR IV report. During a guest visit of Dr. Ladislav Tomásek from the Institute of Hygiene and Epidemiology in Prague procedures have been defined and algorithms have later been developed that are applicable to the Czechoslovakian data and are largely - but not entirely - analogous to procedures used in the BEIR IV report. This work will be continued, and in an extension the methods will be applied to the Czechoslovakian data.
The continued discusson of new risk estimates after the revision of the Japanese dosimetry has emphasized the need to coordinate the methods of analysis in different epidemiological studies, and this has been one of the main aims of the project. The development of joint algorithms has also been important and continues to be important with regard to the need to supplement the conclusions from epidemiological studies with the data from animal experiments. The increasing difficulties of performing animal experiments and the resulting need to combine the efforts of different laboratories have made the use of consistent and comparable methods particularly important.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

- Gesellschaft für Strahlen- und Umweltforschung, Institut für Strahlenschutz, Neuherberg
- Eye Hospital of the University of Munich
- National Cancer Institute/NIH, Radiation Epidemiology Branch, Bethesda/MD, USA
- CEN, Fontenay-aux-Roses
- Institute of Hygiene and Epidemiology, Prague

V. Publications:

Kellerer, A. M.:
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Mathematical methods and models for radiation carcinogenesis studies.
Leukemia Res. **10**, 711-717 (1986)

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A proportional hazards analysis of bone sarcoma rates in German 224-Radium patients.
Strahlenther. **Suppl. 80**, 32-37 (1986)

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In: Cancer Risks (Ed.: P. Bannasch). Heidelberg: Springer-Verlag, 143-153 (1987)

Chmelevsky, D., Mays, C. W., Spiess, H., Stefani, F. H., Kellerer, A. M.:
An epidemiological assessment of lens opacifications with impaired vision in patients injected with Radium-224.
Radiat. Res. **115**, 238-257 (1988)

Kellerer, A. M.:
Strahlenexposition während der Schwangerschaft.
In: Prophylaxe in der Schwangerschaft, Stillen und Kinderernährung (Hrsg.: H. Spiess). Marburg: Deutsches Grünes Kreuz, 111-128 (1988)

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In: Proc. Intern. Colloquium "Epidemiological Investigations on the Health-Effects of Ionizing Radiation". Köln: Institut für Strahlenschutz, 31-41 (1988)

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Experientia **45**, 13-21 (1989)

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Griebel, J.:
Vergleich verschiedener Modelle zur Beschreibung der Zeit- und Dosisabhängigkeit von Tumorraten. Eine Untersuchung zur Mammatumorgenese durch Neutronen- und Röntgenstrahlung niedriger Dosis bei Ratten.
Thesis, University of Würzburg (1989)

RADIATION PROTECTION PROGRAMME
Final Report

Contractor:

Gesellschaft für Strahlen-
und Umweltforschung mbH
Ingolstädter Landstr. 1
D-8042 Neuherberg

Contract no.: BI6-D-221-D

Head(s) of research team(s) [name(s) and address(es)]:

Prof. Dr. W. Gössner
Institut für Pathologie
GSF
Ingolstädter Landstr. 1
D-8042 Neuherberg

Prof.Dr. A.M. Kellerer*
Prof.Dr. H. Spiess*

Telephone number: 089-3187 2312

Title of the research contract:

Investigation of the cataract incidence in the German Radium-224 patients.

List of projects:

1. Epidemiological investigation on radiation cataract by the ophthalmological examination of patients who had received Ra-224.

*This research programme is carried out in coordination with the "Institut für Medizinische Strahlenkunde der Universität Würzburg", Prof. A.M. Kellerer and the "Kinderpoliklinik der Universität München", Prof.Dr. H. Spiess.

Title of the project no.: 1

Epidemiological investigation on radiation cataract by the ophthalmological examination of patients who had received ^{224}Ra

Head(s) of project:

Prof. Dr. H. Spiess

Scientific staff:

Prof. Dr. F. H. Stefani, J. P. Scharff

I. Objectives of the project:

A serial examination provides a possibility to detect cataracts at an early stage where they can be distinguished clinically from spontaneous cataracts or cataracts associated with the original disease that led to the radium treatment. An important feature of this ophthalmologically oriented follow-up will be the repeated examination at regular time intervals of the same patients to assess the evolution of the radiation-induced cataracts. The aim is to gain further insight into the time and dose dependences.

II. Objectives for the reporting period:

Continuing the examinations of patients who were under 20 years of age at first injection with ^{224}Ra.
Some patients with subcapsular cataracts were examined by Prof. Dr. Hockwin (Eye Hospital Bonn) with a "Scheimpflugkamera".

III. Progress achieved:

Earlier in their follow-up of the ^{224}Ra patients Spiess, Mays, and Stefani had recognized an increased incidence of opacities. Since 1969 the questionnaires sent to the patients have included questions on their visual acuity, on visual problems, and on the address of their eye doctor.

By detailed slit-lamp examination of 58 patients it was possible to document bilateral disk shaped paracentral posterior cataracts with a narrow but optically clear subcapsular zone. This clear zone of about 0.5-0.6 mm corresponds to the lens fibres deposited during the period of about 40 years from treatment to examination. These findings established the radiation related origin of these cataracts. Such diagnoses are, however, only possible for non mature cataracts, i. e. cataracts at an early stage that do not yet interfere with vision.

Most of the cataracts recorded among the patients were diagnosed at an advanced stage when the patients had problems with vision and consulted an eye doctor. The cataracts could therefore not be attributed, in the indivual cases, to the radium treatment; especially among the older patients, some were age related. A mathematical analysis of the mature cataracts was performed. The dose and time dependencies of the incidence of cataracts were derived. Critical to the analysis were reliable informations on the time after treatment of the cataract diagnoses. The analysis showed that most of the cataracts diagnosed before the age of 53 were radiation-induced.

No difference in sensitivity with age at treatment could be detected. In agreement with other studies it was found that the data are consistent with either a linear dose relation with threshold or a quadratic relation. Another important conclusion was that a substantial part of the radiation-induced cataracts were diagnosed 40 years after the radium injection or later.

On the basis of these results it was decided to start a systematic ophthalmic investigation of the younger patients, i. e. the patients who were under 20 years of age at treatment. Most of these patients have not yet reached the age with high incidence of senile cataracts. The detailed investigations are therefore still feasable. It is the aim of this complementary investigation to salvage a maximum of information on the incipient lens opacifications and on their evolution in the younger patients.

Of the 128 living patients 54 were born in the 1940s and are now between 41 and 50 years old, 65 were born in the 1930s, and nine were born in the 1920s. 38 out of a total of 218 are known by 1989 to have opacification. This number includes four patients with confirmed cataract who have died and two who had had

surgical cataract extraction at the age of 14 and of 45. In one patient the lens had been studied at autopsy.

58 patients have been examined in detail with a slit-lamp either in the eye hospital in Munich or by the patient's eye doctor. 34 patients were found to have some kind of lens opacity. In 14 out of the 34, the cataracts appeared to be morphologically similar to age-related cataracts. In 14 patients there was a bilateral posterior subcapsular cataract. In eight patients this was a bilateral dense round plaque with a clear subcapsular zone of about 0.5-0.6 mm.

Detailed objective and reproducible documentation of cataract morphology is only possible with Scheimpflug photography including image analysis. A cooperation has been started with the University hospital in Bonn: four patients have had an examination with the Scheimpflugkamera in Bonn by Prof. Dr. Hockwin. The possibility to acquire a Scheimpflugkamera by an additional research grant that will include also heavy ions research is at present being explored.

Table 1: SUMMARY OF CATARACTS IN THE ^{224}Ra PATIENTS IN PROJECT 2 (Dec. 1989)

Patient No.	Category	Total
1- 975	Total Adults	47
1101-1117	Boys, 1- 5 y	5
1201-1235	Boys, 6-10 y	3
1301-1328	Boys, 11-15 y	4
1401-1431	Boys, 16-20 y	6
	Total Boys	18
1501-1517	Girls, 1- 5 y	3
1601-1635	Girls, 6-10 y	6
1701-1725	Girls, 11-15 y	5
1801-1830	Girls, 16-20 y	6
	Total girls	20
	Total juveniles	38
	Total patients	**85**

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

- Prof. Dr. C. W. Mays, National Cancer Institute/NIH, Radiation Epidemiology Branch, Bethesda/MD, USA; deceased in 1989

- Dr. D. Chmelevsky, Gesellschaft für Strahlen- und Umweltforschung, Institut für Strahlenschutz, Neuherberg

- Prof. Dr. Hockwin, Eye Hospital, University of Bonn

V. Publications:

1. Chmelevsky, D., Mays, C. W., Spiess, H., Stefani, F. H., Kellerer, A. M.:
An epidemiological assessment of lens opacifications with impaired vision in patients injected with Radium-224.
Radiat. Res. **115**, 238-257 (1988)

2. Stefani, F. H., Spiess, H., Mays, C. W.:
Cataracts in patients injected with a solution of Radium-224, colloidal platinum, and the red dye eosin (Peteosthor).
In: BIR Report No. 21, Risks from Radium and Thorotrast (Eds.: D. M. Taylor, C. W. Mays, G. B. Gerber, R. G. Thomas). London: British Institute of Radiology, 12-21 (1989)

3. Chmelevsky, D., Mays, C. W., Spiess, H., Stefani, F. H., Kellerer, A. M.:
The cataract response in Radium-224 patients.
In: BIR Report No. 21, Risks from Radium and Thorotrast (Eds.: D. M. Taylor, C. W. Mays, G. B. Gerber, R. G. Thomas). London: British Institute of Radiology, 21-25 (1989)

4. Stefani, F. H., Mays, C. W., Spiess, H.:
Strahlenkatarakt nach Injektion von Radium-224.
Fortschr. Ophthalmol. **86**, 32-37 (1989)

RADIATION PROTECTION PROGRAMME
Final Report

Contractor:

Gesellschaft für Strahlen-
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Ingolstädter Landstr. 1
D-8042 Neuherberg

Contract no.: BI6-D-085-D

Head(s) of research team(s) [name(s) and address(es)]:

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Institut für Strahlenbiologie
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Ingolstädter Landstr. 1
D-8042 Neuherberg

Telephone number: 89-3187 2250

Title of the research contract:

Molecular and cellular mechanisms of neoplastic cell transformation.

List of projects:

1. Molecular and cellular mechanisms of neoplastic cell transformation.

Title of the project no.: BI 6 - 085 - D

Molecular and cellular mechanisms of neoplastic cell transformation

Head(s) of project:

Prof. Dr. Dr. Ulrich Hagen
Institut für Strahlenbiologie der GSF
D 8042 Neuherberg

Scientific staff:

Dr. Cornelia Morawetz	(100 %)
Dr. Wolfgang Vogel	(100 %)
Prof.Dr. Ulrich Hagen	(25 %)
Prof.Dr. Klaus-Rüdiger Trott	(15 %)

I. Objectives of the project:

Among other mechanisms for radiation induced cell transformation, the transposition (translocation) of oncogenes and the movement of viral enhancer elements have been discussed. As a model for the inducibility of transposition of mobile gene elements the behaviour of Ty-elements in yeast cells will be studied after treatment with mutagenic agents. In addition, cell transformation of mammalian cells (C3H/T1/2) will be tested with respect to the dose effect relationships as modified by fractionation and low dose rate, in order to learn more about the mechanism of subtransformational repair.

II. Objectives for the reporting period: (July 1985 - Dec.1989)

a) Studies on the mechanisms of Ty-insertion into the promotor regions of the yeast ADH2- and ADH4-gene in chromosomal and in episomal DNA. Effect of mutagenic agents as gammarays, ultraviolet light and ethylmethanesulfonate (EMS) on the frequency of transposition and its dependence on the metabolism of the cell.

b) Repair kinetics of the subtransformational damage of C3H/10T1/2 cells. Experiments with fractionated irradiation and with low dose rates and in dependence of the state of proliferation.

III. Progress achieved:

a) INDUCED TRANSPOSITION OF TY-ELEMENTS IN YEAST.

1. Methodology:

To study transposon-mediated mutations in eukaryotic organisms the yeast Saccharomyces cerevisiae was used. Haploid cells can be used, even recessive mutations are expressed phenotypically. S.cerevisiae is a facultative anaerobic organism, i.e. it can grow in the presence (oxidative pathway) of oxygen as well as in its absence (fermentational pathway). These pathways are regulated very accurately, as for instance the isoenzymes of alcohol dehydrogenase (ADH I, II, III and IV). Strains of S. cerevisiae that carry a mutated ADH1 gene (adh 1) are unable to grow fermentatively on media containing glucose. If in addition the respiratory chain is blocked by antimycin A, no growth at all is possible on glucose containing media.

Ty-integration into the promotor regions of the ADH2- and ADH4 gene can lead to the expression of the glucose-repressed ADH2 gene and to the expression of the normally silent ADH4 gne. Fermentation is possible again. Thus Ty-integrations are selectable by looking for mutants able to grow under these conditions (glucose and antimycin A). (Figure 1) However, besides the integration of Ty-elements

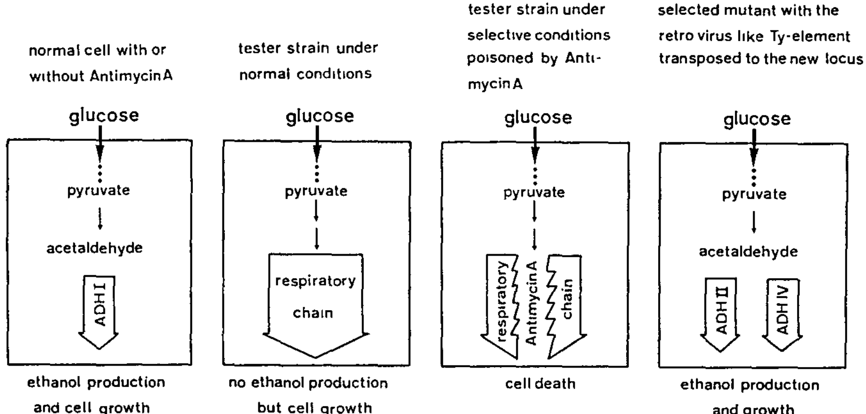


Figure 1.

other mutations lead to the same phenotype. Therefore further analysis of the mutants (search for altered restriction patterns at the ADH2- and ADH4 gene) is done to evaluate the true number of transpositions events.

2. Results and Discussion.

With this test system, various aspects of induced transposition were studied:

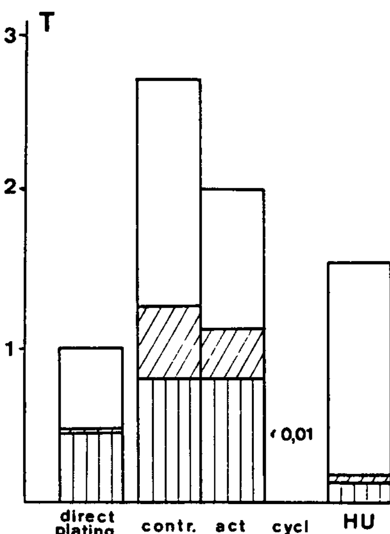
i) Dose effect relations after exposure to mutagenic agents (Morawetz and Hagen, 1990):

The efficiency of agents to induce transposition can be evaluated by relating the transposition number to the lethal hits. (Yield of transposition per treated cell). The alkylating agent EMS is much more effective than UV-light or even gamma irradiation in this test system. The dose effect curves correspond to that of the yield of mutation, i.e. an increase with increasing doses and then a decrease at higher cytotoxic doses (Morawetz 1987). It could be shown, that transpositions to the ADH4-locus are more frequent than transpositions to the ADH2 locus, depending on the type of agens and of the strain used.

ii) Effect of inhibitors on Ty-transposition:

Incubating gamma-irradiated cells for several hours leads to an fourfold increase in transposition yield up to a plateau at 8 hours after irradiation. Inhibiting DNA synthesis by hydroxyurea prevents this increase; inhibiting protein synthesis by cycloheximide abolishes spontaneous and induced transposition completely. However, inhibition of RNA-synthesis by actinomycin D is much less effective, the increase of the transposition yield will only be delayed (Morawetz und Hagen, 1990) (Figure 2).

Fig. 2: Transposition to the ADH loci in strain MC31 after treatment with metabolic inhibitors. Gamma-irradiation with 100 Gy, Four hours incubation without (Control) or with actinomycin D, cycloheximide or hydroxyurea.
long barred areas: ADH4 locus; cross hatched: ADH2 locus
open areas: mutation with no detectable insertion.



iii) Expression of Ty-coded mRNA after mutagenesis (unpublished results).
After treatment of the yeast cells, mRNA was isolated and hybridized with a labelled Ty-epsilon fragment. Ty-specific RNA was quantified in relation to the actine-RNA. In addition to the wild type also a variety of radiosensitive mutants (rad 1, 2, 5, 6 and 12) were studied in this way (unpublished results). Under certain conditions, there is an increase of Ty-specific mRNA-synthesis in gamma-irradiated or UV-irradiated cells. This effect was especially strong in the rad-mutants, whereas EMS inhibited new Ty-mRNA-synthesis. Because of the long halflife of Ty-mRNA (2,5 hours), it was not possible to proof, that induced transposition of Ty-elements after mutagenic treatment is mediated by an induced synthesis of Ty-mRNA.

iv) Transposition in diploid cells. (unpublished results)
The previous experiments were performed with haploid cells; it was of interest, whether induced transposition will occur also in diploid cells. Special strains had to be constructed

by selective pairing to follow transposition of Ty-elements into either allele of ADH2 or ADH4. The results did show, that the transposition yield in diploid cells is much lower than in haploid cells, although both alleles are affected. As in haploid cells insertion into the ADH4-locus is more frequent than into the ADH2-locus. The highest yields were observed at about 30 - 50 percent survival of the cells; higher doses apparently damage the transposition mechanism.

v) Transposition of Ty-elements into plasmids (Vogel, 1989). The results reported dealed with induced transposition into loci of chromosomal DNA. Such experiments, however, don't allow an analysis of the integration positions in detail. For such a study Ty-insertions into recoverable plasmid DNA have to be analysed. Several strains were constructed with destroyed copies of the chromosomal ADH2- and ADH4-gene. In addition, plasmids were constructed carrying the ADH2- and or ADH4-gene and introduced into the cells described. Mutants selected for antimycin A resistance after treatment, would indicate a Ty-insertion in the plasmid DNA. Such insertions were found in the 5'-position of the ADH2- or ADH4-gene. The exact position of Ty-insertion was determined by restriction analysis and DNA sequencing (Figure 3).

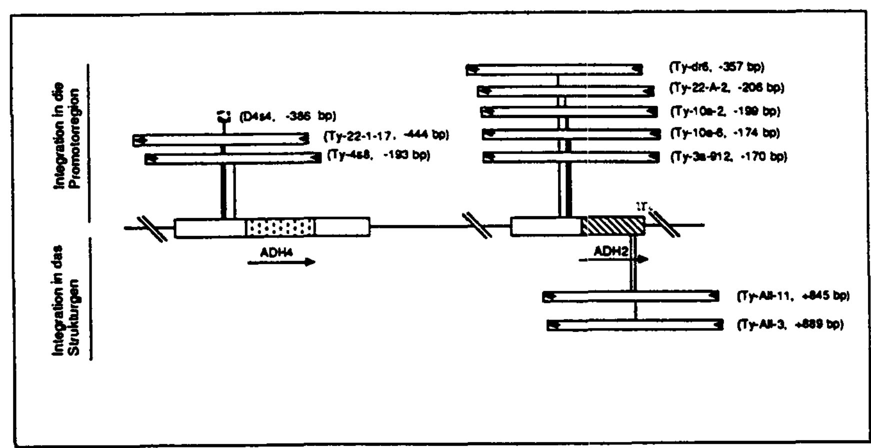


Figure 3: Positions of Ty-insertions at the ADH2- and ADH4 locus.

Comparing these data derived from plasmids with data in the literature on integration sites in chromosomal DNA, no differences were observed. Integrated Ty elements on plasmids showed the the same orientation of the Tyelement relative to the structural gene, the same distribution of integration sites and corresponding cis-activation. Comparing the DNA sequence in the neighbourhood of 16 integration sites in the ADH2 and ADH4-gene no sequence specifitiy for the integrations could be observed. However, all of the integrations 5' to the ADH2 gene are located within 86 bp, indicating a biased selection of mutants by antimycin A resistance. Possibly the "upstream activation sequence" of the ADH2 promotor is preventing cis activation of the ADH2 gene by Ty-elements situated beyond this position. Furthermore, also six mutants were selected where a solo-delta-element in the 5'position of the ADH4-gene leads to gene expression - a hitherto unknown phenomenon.

3. General discussion about transposition of Ty-elements.

The results described in this chapter clearly show the inducibility of Ty-transposition in yeast by mutagenenic respectively carcinogenic agents. Details of the mechanism of induced transposition of gene elements were studied. If - as pointed out repeately - malignant cell transformation is due also to transposition of mobile gene elements, especially retroviruses, it is necessary to develop a corresponding system in mammalian cells as in yeast in order to study the relation between transposition and carcinogenesis, induced by radiation and chemicals.

b) RADIATION INDUCED TRANSFORMATION IN MAMMALIAN CELLS.

1. Methodology:

The influence of protracting low LET irradiation from acute exposures of less than 10 minutes duration to those lasting hours and days (up one week at 1 Gy/d) on the transformation frequency was studied in C3H10T 1/2 cells. Cells were plated in T-30 flasks at a density of less than 500 clonogenic cells (i.e. 2,000 cells, mean plating efficiency 22%) or less than 500 colony forming survivors per flask. Irradiation from a Co60 source (Eldorado) was started 16 hours after plating. The dose rate for acute irradiation was 1 Gy/min. Low dose rate irradiation was performed by putting an incubator into the irradiation room, the required dose rate was achieved by adjusting the distance to the source and by adding lead filters close to the source. Dose rate and dose distribution in the incubator were measured by an ionisation chamber in each position prior to the respective experiment. The longest exposure time was 8 days at a dose rate of 1 Gy per day. 10 days after irradiation cell survival was determined by counting colonies which had developed from the plated cells in 6 flasks. The other flasks remained in the incubator for a further six weeks with medium changes every 10 days. The number of transformed foci (type II and III together) per surviving, clonogenic cell was determined in the experiments for doses of 2, 4, 6 and in some experiments 8 Gy.

2. Results and Discussion.

The spontaneous transformation frequency was very low in our experiments, it varied over the years but was always below 10^{-5}. Acute irradiation with a dose rate of 1 Gy/min increased the mean transformation frequency to

$5. 10^{-5}$ at 2 Gy
$12. 10^{-5}$ at 4 Gy
$35. 10^{-5}$ at 6 Gy
and $40. 10^{-5}$ at 8 Gy.

The 4 Gy value is based on more than 100 transformations and over a period of 3 years transformation frequency ranged between different series of experiments from $8. 10^{-5}$ to $20. 10^{-5}$. There was a decrease in transformation frequency by a factor of between 2 and 3 as the dose rate was decreased to 1 Gy/h at dose between 4 Gy and 8 Gy. Further protraction lead to even greater reduction in transformation frequency. Recent experiments using a dose rate of 1 Gy/d yielded a dose rate reduction factor of 2 compared to 1 Gy/h or a dose rate reduction factor of 5 compared to 1 Gy/min. There was no significant difference in transformation frequency between 1 Gy/d and 4 Gy/d.

In order to rule out the possibility that the further reduction in transformation frequency by protracting irradiation over several days was due to a decrease in transformation sensitivity as cells divide after plating, experiments were performed with high dose rate irradiation given either 6h, 16h, 24h or 48h after plating but no significant difference in transformation frequency was found (These time span the duration of all exposures at 4 Gy/d which gave similar results as 1 Gy/d).

Therefore, we conclude that there is a significant sparing effect on cell transformation at very low dose rates, i.e. those dose rates at which no furter sparing effect with regard to cell survival can be found.

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V. Publications:

1. C. Morawetz: Effect of irradiation and mutagenic chemicals on the generation of ADH2-constitutive mutants in yeast. Significance for the inducibility of Ty transposition.
   Mutation Research 177, 53-60 (1987)

   C. Morawetz, U.Hagen: Effect of irradiation and mutagenic chemicals on the generation of ADH2- and ADH4-constitutive mutants in yeast. 2. The inducibility of transposition by UV and ethyl methane sulfonate and the influence of metabolic inhibitors on the event.
   Mutation Research, in press

   C. Morawetz: Transposition of retrotransposons after gamma and UV irradiation.
   In: Terrestrial Space Radiation and its Biological Effect: (Eds.: P.C. McCormack, C.F. Swenberg, H. Bücker), New York London: Plenum Publ. Corp., 345-350 (1988)

2. W. Vogel: Entwicklung eines Systems zur Isolierung von Ty-integrationen mit dem ADH2 und ADH4 Gen auf Plasmiden in Saccharomyces cerevisiae. - Charakterisierung der Integrationen.
   Dissertation, München, Ludwig-Maximilians-Universität, 1989

   K. Müller: Die Entstehung transformierter Zellen nach fraktionierter Bestrahlung in vitro.
   Diplomarbeit, Fakultät für Biologie, Ludwig-Maximilians-Universität München, 1987

   R. Schade: Integration von Ty-elementen in der Promotorregion des ADH2 gens von Hefe
   Diplomarbeit, Fakultät für Biologie, Ludwig-Maximilians-Universität München, 1990

RADIATION PROTECTION PROGRAMME
Final Report

Contractor:

Contract no.: B16-D-095-UK

Berkeley Nuclear Laboratories
Central Elect. Generating Board
Berkeley
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Title of the research contract:

Filtered neutron beam studies (biological effects).

List of projects:

1. Filtered neutron beam studies (biological effects).

Title of the project no.: BI6-D-095-UK

Filtered Neutron Beam Studies (Biological Effects)

Head(s) of project:
Dr A.J. Mill

Scientific staff:
Ms S.C. Hall
Dr A.J. Mill
Dr J. Wells

I. Objectives of the project:

To undertake a wide-ranging study of the biological effects of intermediate-energy neutrons and assess implications for radiological protection and radiobiology.

II. Objectives for the reporting period:

To establish the relative biological effectiveness of 24 keV neutrons for:
(i) cell transformation _in vitro_ in C_3H 10T½ mouse fibroblasts; and
(ii) micronucleus induction in human peripheral blood lymphocytes.

III. Progress achieved:

1. Introduction

Little biological data exist for exposure to intermediate-energy neutrons, yet a knowledge of the relative effects of these neutrons is important. This is because they contribute a significant proportion of the occupational dose equivalent around nuclear reactors and other neutron-producing facilities such as medical accelerators and high energy synchrotrons (Harvey and Beynon, 1972; Mill, 1982). However, the neutron dose equivalent includes a factor, the quality factor (Q), to account for the increased effectiveness of neutrons compared with low-LET radiations in producing late biological effects such as cancer and recently, national bodies such as the National Council on Radiation Protection and Measurements (NCRP) (in the USA) and the National Radiological Protection Board (NRPB) (in the UK) have suggested an upward revision of Q values. The case for these revisions is based almost exclusively on evidence from experimental data since no significant epidemiological data for neutron exposures are available. However, these data are for fission-energy or fast neutrons - high energies not often encountered in occupational exposure. Information on the relative effects of intermediate-energy neutrons would be more relevant and should form a major input into decisions concerning possible revisions in Q values.

Also of interest is that the ionising products of intermediate-energy neutrons have ranges in tissue which are comparable to the dimensions of cellular components thought to be key targets in the expression of radiation damage. Thus data on the effects of these neutrons will aid fundamental studies aimed at understanding the mechanisms of radiation-induced injury. Such studies complement the work carried out with soft X-rays (Cox et al., 1977).

Finally, a knowledge of the biological effects of intermediate-energy neutrons is crucial for their application to neutron capture therapy (NCT). Following the recent successes reported from Japan on the treatment of high--grade gliomas with thermal neutrons (Hatanaka, 1983), there is now renewed interest world-wide in this technique (Mill, 1989). However, thermal neutrons

have little penetrating power and for adequate irradiation of deeply-sited tumours part of the patient's skull must be removed. Intermediate-energy neutrons, however, offer a superior treatment modality over thermal neutrons (Mill and Harrison, 1988; Perks et al., 1988). They should permit the uniform irradiation of all tumours using multifraction treatments without recourse to surgery. For their ultimate application to NCT the biological effects of intermediate-energy neutrons needs to be ascertained.

This report details experiments carried out during the past four years to determine the relative biological effectiveness of intermediate-energy neutrons. Two cell lines (HeLa S3 human cancer cells and C_3H 10T½ mouse fibroblasts) and fresh human blood were used in the irradiations. The biological endpoints investigated were survival (HeLa and C_3H 10T½ cells), cell transformation (C_3H 10T½ cells) and micronucleus induction (human peripheral blood lymphocytes).

2. The neutron beams at the Harwell Laboratory

One method of producing beams of intermediate-energy neutrons is to filter the broad spectrum of neutrons present in a nuclear reactor. For example, a filter composed of iron, aluminium and sulphur can be used to produce relatively pure beams of 24 keV neutrons and this technique has been used on a number of research reactors (Mill and Harvey, 1980). Unfortunately, many beams are unsuitable for radiobiological experiments because of their low intensity or because of their high contribution from fast neutrons. The fluence-to-dose conversion factor changes quite rapidly with neutron energy in the region from 10 to 100 keV and it is important to keep fast neutron contamination to a minimum.

In 1985, an intense and pure beam of 24 keV neutrons, suitable for radiobiological studies was made available on the PLUTO high-flux research reactor at the Harwell Laboratory. The dosimetry and spectrometry of the beam are described by Perks et al. (1988). The free-in-air kerma rate from neutrons is 0.35 Gy h^{-1} with 79% of the kerma due to neutrons at 24 keV (the remaining 21% is from neutrons with higher energies, although these represent only 7% of the total fluence). The contribution from gamma radiation is 0.03 Gy h^{-1}.

Fortunately, the beam is a vertical one, enabling the irradiation of cell cultures in petri dishes.

Most of the irradiations described in this report were carried out using the 24 keV neutron beam. However, a small number of exposures using a beam with a mean energy of 10 keV were also carried out. This beam is produced by using a filter of aluminium, argon and sulphur. Unlike the 24 keV beam this beam is not truly monoenergetic.

3. Experimental Procedure

Two cell lines (HeLa S3 human cancer cells and C_3H 10T½ mouse fibroblasts) and fresh human blood were used in the irradiations. The procedures for both HeLa and 10T½ cells were similar. Approximately six days prior to irradiation sufficient numbers of cells were inoculated into 35 mm diameter petri dishes containing 1.5 cm^3 of growth medium and allowed to grow to confluence (in the case of HeLa) or near confluence (for 10T½). At this stage the growth rates of the cultures are greatly reduced. This was necessary to suppress proliferation during the long irradiation times which were up to 18 hours. The irradiations were carried out at 37 ^oC, maintained by blowing air over the specimen. In order to prevent evaporation of the medium during exposure the dishes were sealed with thin plastic film.

Following removal from the neutron beam the medium was sucked off and trypsin added to detach the cells from the surface of the dish. The cells were then resuspended in about 5 cm^3 of growth medium and the cell concentration evaluated. The suspensions were diluted and replated onto fresh 10 cm diameter petri dishes (HeLa cells) or into prewarmed and pregassed 150 cm^2 flasks containing 50 cm^3 of growth medium (10T½ cells). The samples were transported to Berkeley Nuclear Laboratories (BNL) in a battery-powered portable incubator. Here they were transferred to a warm room kept at 37 ^oC.

HeLa cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 16% foetal calf serum (FCS), 1% non-essential amino acids and 2 mM HEPES. For 10T½ cells the culture medium was Eagle's Basal Medium

supplemented with 10% heat-inactivated FCS and maintained in an atmosphere containing 5% carbon dioxide.

For the survival assay, samples were fixed and stained with methylene blue twelve or thirteen days after exposure. Surviving fractions for both cell lines were assessed on the usual criterion of fifty or more non-giant cells per colony.

For cell transformation samples were incubated for a further four weeks with weekly medium changes. After the first medium change the serum concentration was reduced to 5%. After two to three weeks incubation 10T½ cells have formed a confluent contact-inhibited monolayer on the base of the flasks. Transformed cells grow into dense piles (known as foci) and are tumourogenic. Foci were scored by eye on the basis of morphological changes. The spontaneous transformation frequency in unirradiated cells in our laboratory is about 10^{-5}.

Cells were either irradiated free-in-air (both 10T½ and HeLa, using 24 keV neutrons) or at various depths within a polyethylene phantom (HeLa only, using 10 keV and 24 keV neutrons). For the phantom irradiations up to four dishes were placed at various depths within a 60 mm diameter phantom and exposed to total incident neutron fluences in the range 0.8 to 2.4×10^{12} cm^{-2}. For some phantom irradiations boric acid (enriched in ^{10}B) was added to the medium to give ^{10}B concentrations in the range from 30 to 80 $\mu g\ ml^{-1}$. Cells so irradiated are additionally exposed to 1.5 MeV α-particles (range in tissue about 9 μm) produced by the reaction:

$$^{10}B + {}^{1}n \Rightarrow {}^{7}Li + \alpha + 2.79\ MeV.$$

In the case of the blood cultures, these were irradiated as whole blood within a few hours of being obtained from a healthy donor. After transportation to BNL, growth medium (Eagles Minimum Essential Medium plus heat--inactivated FCS) and phytohaemagglutinin were added to the blood which was then cultured for forty-four hours. At this time cytochalasin B was added and the incubation period continued for a further twenty-eight hours when the cultures were fixed and stained. This procedure results in the formation of a

number of binucleated lymphocytes, some of which will also contain small fragments of nuclear material (micronuclei). The cytochalasin B suppresses daughter cell separation but allows normal nuclear division. This provides an accurate tally of lymphocytes which have undergone one division and which can potentially express micronucleus formation. Further details of this novel technique are described by Hall and Wells (1988).

For the determination of the relative biological effectiveness (rbe) of the neutron beam, X-ray irradiations were also carried out. For these a Philips 420kV X-ray set running at 250 kV and 15 mA with 1.6 mm aluminium and 1.6 mm copper filtration was used. At a distance of 78 cm from the focus the absorbed dose-rate in tissue is 0.4 Gy min^{-1}.

4. Results and discussion

4.1 Survival data

The free-in-air survival curves obtained after irradiation with 24 keV neutrons are exponential with no evidence of a shoulder. This kind of survival curve is typically found for irradiation with densely-ionising radiation. Until these experiments were carried out it was not certain whether the low energy recoil protons produced by 24 keV neutrons would act like sparsely- or densely-ionising radiation. These recoil protons have a maximum range of about 500 nm and the implication is that interactions between events greater than this do not contribute to cell death. The interpretation of data obtained with 24 keV neutrons using V79 cells is discussed in detail by Holt (1988).

The survival curves have been fitted to the data by a non-linear least squares regression analysis using the equation:

$$S = \exp(-\alpha D - \beta D^2)$$

where S is the surviving fraction and D is the absorbed dose. For the 24 keV neutron curves, the value of β was taken as zero. The calculated values of α and β are given in table I, along with the low-dose rbe, calculated as the ratio of α values.

Table I. Parameters of the best-fit lines calculated by least-squares regression for the relationship: $S = \exp(-\alpha D - \beta D^2)$ for HeLa and C_3H 10T½ cells.

Cell line	250 kVp X-rays α/Gy^{-1}	β/Gy^{-2}	24 keV neutrons α/Gy^{-1}	rbe (ratio of α values)
HeLa S3	0.410 ± 0.059	0.045 ± 0.014	1.35 ± 0.04	3.3 ± 0.5
C_3H 10T½	0.337 ± 0.035	0.007 ± 0.005	1.13 ± 0.04	3.4 ± 0.6

Cells irradiated in the phantom are exposed to a wider quality of radiations. These are (i) α-particles and lithium recoil nuclei (boronated cells only); (ii) protons from elastic scattering events with hydrogen and capture events in nitrogen; and (iii) gamma rays, both incident in the beam and from capture events in hydrogen. The contribution of each of these components and their variation with depth in the phantom have been calculated using Monte--Carlo computer code techniques (Mill and Harrison, 1988). The data set containing 79 dose-survival points were then analysed by multiple linear regression on the assumption that (i) the damage incurred from the three components was independent and (ii) the dose-response relationship for each component was exponential. ie the data were fitted to the equation:

$$\log(\text{survival}) = k_p D_p + k_\alpha D_\alpha + k_\Gamma D_\Gamma$$

where the subscripts p, α and Γ refer respectively to proton recoil, α-particle and gamma ray components. D_{37} values ($=-k^{-1}$) of 0.84 ± 0.04 Gy, 0.78 ± 0.04 Gy and 10.3 ± 5.4 Gy were obtained.

The value k_p of 1.28 Gy^{-1} is in excellent agreement with the value of α for the free-in-air 24 keV neutron exposures of 1.35 Gy^{-1} as given in Table I. Clearly there is essentially no difference in the biological effectiveness between 24 keV neutrons and internally-emitted 1.5 MeV α-particles. The linear energy transfer (LET) of α-particles with this energy is approximately 200 keV μm^{-1} (Morgan et al., 1988; Mill et al., 1989).

4.2 Micronucleus data

The frequency of micronucleus induction as a function of radiation dose can be fitted to the relationship (Wells et al., 1989):

$$N = a + bD + cD^2$$

where N is the number of micronucleui per binucleate cell and D is the absorbed dose. A summary of the results obtained for 24 keV neutrons, α-particles of LET 23 keV μm^{-1} and 250 kVp X-rays is shown in Table II.

Table II. Parameters of the best-fit lines calculated by least-squares regression for the relationship: $N = a + bD + cD^2$ for micronucleus induction in human lymphocytes.

Radiation Type	a $/10^{-3}$	b $/10^{-3}$ Gy^{-1}	c $/10^{-3}$ Gy^{-2}	rbe (ratio of b values)
250 kVp X-rays	29 ± 6	44 ± 23	55 ± 6	1
23 keV μm^{-1} α-particles	20 ± 13	330 ± 54	-	7.5 ± 4.1
24 keV neutrons	16 ± 6	930 ± 55	-	21 ± 11

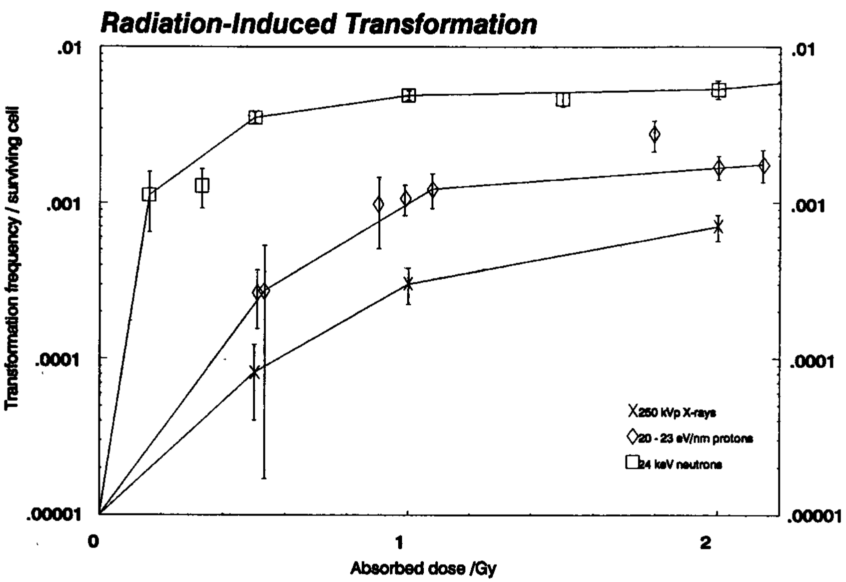
The rbe obtained for 24 keV neutrons is in broad agreement with the value reported by Lloyd et al., (1988) for chromosome aberrations, also in human blood.

4.3 Transformation data

Only a preliminary analysis of the transformation data has so far been carried out due to the large number of samples to be scored and analysed. The figure shows the results for irradiation with 24 keV neutrons, 20 and 23 keV μm^{-1} α-particles and protons and 250 kVp X-rays. Clearly 24 keV neutrons are very effective in producing transformants. At a transformation frequency equivalent to that produced by 2 Gy of X-rays, the rbe is about 12. For doses above about 0.5 Gy of neutrons it appears that the transformation frequency is higher than any level observed with X-rays, thus it is impossible to define a value for the rbe at these neutron doses.

5. Summary

The relative biological effectiveness of 24 keV neutrons in a range of biological systems has been shown to be high and comparable to the effectiveness of high-LET α-particles. However, this response is well predicted by the



LET-distribution of 24 keV neutrons and is in broad agreement with the currently-used values of the quality factor for intermediate-energy neutrons.

5. Acknowledgements

We are indebted to staff at the Harwell Laboratory for their support and the use of their facilities; also to Dr L. Heiber for supplying our stocks of C_3H 10T½ cells and advice on their culture.

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor:

Centre d'Etude de l'Energie
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Contract no.: BI6-D-090-B

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Title of the research contract:

Mechanism of radiation-induced leukemogenesis and osteosarcomagenesis.

List of projects:

1. Mechanisms of radiation-induced leukemogenesis.
2. Molecular biology of radiation-induced osteosarcomagenesis. Role of oncogenes and viruses.

Title of the project no.: 1

Mechanisms of radiation-induced leukemogenesis

Head(s) of project:

M. Janowski

Scientific staff:

M. Janowski
B. Borremans
R. Hooghe

I. Objectives of the project:

For three decades, retroviruses have been suspected -although not demonstrated unequivocally- to play a role in radiation-induced thymic lymphomagenesis in mice. Our proposal consisted in determining the molecular mechanisms by which radiation leukemia virus (RadLV) exerts its leukemogenic effect, and in searching if similar mechanisms take place in radiation-induced thymic lymphomagenesis. Moreover, we proposed to investigate if the latter involves the participation of oncogenes, activated either by falling under the control of retroviral genomes or other genomic elements, or by other mechanisms such as somatic mutational events.

II. Objectives for the reporting period:

1. Molecular cloning and sequencing of the RadLV proviral genome and identification of the sequences that determine its leukemogenic potential, to disclose the mechanisms by which this potential comes to expression.
2. Search for experimental evidence in favour of the idea that RadLV might activate oncogenes by mutational insertion.
3. Search for acquired RadLV-like proviral genomes and for retrovirus-activated oncogenes in the radiation-induced thymic lymphomas.
4.Identification of factors other than retroviruses or retrovirus-controlled cellular genes, that might contribute to the malignant transformation (i.e. mitogenic growth factors or mutated oncogenes).

III. Progress achieved:

1. Methodology

1.1. Animals and viruses. Inoculations of RadLV were done in rats of the R strain or in mice of the C57BL strain (initially C57BL/Cnb, later C57BL/Ka). In the initial experiments we used the retroviral complex released by the cultivated BL/VL3 cells, and later the molecularly cloned RadLV/VL3(T+L+) virus. Radiation-induced thymic lymphomas were obtained by treating C57BL/Ka mice with 4 weekly doses of 1.75 Gy of X-rays (direct induction protocol). An indirect induction protocol consisted in irradiating, according the same schedule, thymectomised mice bearing the Thy-1.2 surface antigen. Immediately therafter, they received a subcutaneous graft with thymuses from Thy-1.1 bearing congenic newborn mice.

1.2. Molecular cloning of the RadLV genome. Retroviruses released by an in vitro cultivated RadLV-induced thymic lymphoma (RadLV/VL3) were used to infect a permissive cell line (BL-RL12-NP). Newly synthesized proviral DNA was isolated before its integration into the cellular DNA, and cloned into the PstI restriction site of the bacterial plasmid pBR322. The biological integrity of the clone was tested by assessing the release of thymotropic and lymphomagenic virus upon transfection of cultivated cells (SC-1).

1.3. Nucleotide sequencing. Sequencing of the cloned RadLV genome was performed by the chemical method of Maxam and Gilbert. Later, sequencing of oncogenes was performed using the enzymatic dideoxy method. The primers for the Klenow fragment of DNA polymerase I were synthesized chemically using a Cyclone DNA Synthesizer (New Brunswick).

1.4. Molecular hybridizations. Provirus acquisitions and gene rearrangements were investigated using restriction enzyme digestions, agarose gel electrophoresis of the restriction fragments, transfer to filter membranes (Southern technique) and molecular hybridization with adequate probes. Gene expression was investigated by agarose gel electrophoresis of polyadenylated RNA followed by transfer to filter membranes (Northern method) and molecular hybridization with adequate probes received from various sources. Specific detection of RadLV-like proviruses was done with a probe (pEc-B4) that we hade been made able to choose upon examination of the nucleotide sequence of RadLV.

1.5. Polymerase chain reaction (PCR). Whole chromosomal DNA was used as a substrate for the amplification of specific gene fragments. The reaction was primed with chemically synthesized oligodeoxyribonucleotides (Cyclone DNA Synthesizer, New Brunswick), and performed with Taq DNA polymerase in a home-made, computer-driven automated PCR instrument.

1.6. Involvement of growth factors in radiation leukemogenesis. The possible involvement of interleukin 1 (IL-1) and interleukin 6 (IL-6) was studied by inhibiting their synthesis with indomethacin, present at 20 ug/ml in the drinking water from the 8th day before the first irradiation. The possible role of interleukin 2 (IL-2) was investigated by injecting three times a week the immunosuppressive peptide cyclosporin A (25 to 100 mg/kg) from the day before the first irradiation. In situ molecular hybridization was used to detect the expression of specific RNAs. Cytokine levels in blood and culture medium were measured by bioassays.

2. Results.

2.1. Investigations on the viral etiology of radiation-induced lymphomas.

2.1.1. Molecular cloning of the RadLV genome.

Twenty hour cocultures of RadLV-producing BL/VL3 cells with BL/RL12-NP cells displayed, as expected, the appearance of a very homogeneous population of 8.9 kilobase pair (kbp) long extrachromosomal retroviral DNA molecules. Its homogeneity was demonstrated by the fact that digestions with a series of restriction enzymes yielded homogeneous patterns. The molecules were shown not to contain other PstI restriction sites than that present in each of the two identical long terminal repeat (LTR) sequences. Therefore, PstI digestion yielded a 8.3 kbp fragment that was representative of the whole retroviral genome, including one split LTR of which part of the sequence was present at the 5' end and the other part at the 3' end. This fragment was ligated to the bacterial plasmid pBR322, yielding a recombinant plasmid that could be replicated to ponderal amounts after transformation and cultivation of competent bacterial cells.

After its recovery from the plasmid by PstI digestion, the purified insert was used to transfect SC-1 cells which, as soon as a few hours thereafter, showed a massive production of retroviral particles. When inoculated into C57BL/Ka mice, they were shown to be thymotropic (early appearance of retroviral antigens on the surface of the thymic cells, as shown by immunofluorescence) and lymphomagenic (appearance of thymic lymphomas, with the same incidence (100 %) and latency period (3 to 6 months) as the in vitro propagated RadLV/VL3.

Because of the homogeneity of the uncloned extrachromosomal DNA, and because of the thymotropicity and leukemogenicity of the virus released upon transfection, we concluded that we had succeeded in cloning molecularly the major thymotropic and leukemogenic componant of the RadLV complex produced by the BL/VL3 thymic lymphoma cell line. Hence, we called it RadLV/VL3(T+L+).

2.1.2. The nucleotide sequence of RadLV/VL3(T+L+).

The RadLV/VL3(T+L+) genome is 8,318 nucleotides long. It is typically that of a chronic murine leukemia virus in that it contains the LTR, the gag gene (encoding the internal proteins p15, p12, p30 and p10), the pol gene (encoding a protease, the reverse transcriptase and an endonuclease) and the env gene (encoding the membrane glycoprotein gp70 and protein Np15, and the so-called R peptide of which the function is unknown). In contrast with acute murine leukemia viruses, it does not contain sequences derived from a cellular gene.

The bulk of the sequence is very similar to that of the endogenous, ecotropic Akv retrovirus of the AKR mouse. Its recombinant nature is however attested to by the obvious xenotropic features of the LTR and of the R peptide sequences. Outside the LTR, the sequence did not show characteristics that could be tentatively related to the oncogenic potential of the virus.

The LTR is 583 nucleotides long, and contains the typical TATA and CAT box sequences that are the promotors for RNA synthesis. In the 5' LTR, these promotors are responsible for the synthesis of the viral RNAs. The template RNA for the gag and pol gene products is 8,318 nucleotides long. Due to the presence of two potential splicing sites, the template RNA for the env gene products may be either 3,019 or 3,322 nucleotides long. The analysis of the viral RNAs from virus-induced thymic lymphomas indicates that both sites may be used. In the 3' LTR, the promotors for RNA synthesis may play an important role in leukemogenesis. Indeed, they should be capable of promoting the synthesis of RNA from cellular genes adjacent to the 3' end of the integrated provirus. Proviral insertion adjacent to an oncogene might in this way lead to the appearance of a clonal tumor due to overexpression of this oncogene. This had been shown to be the case in a few retrovirus-induced avian or murine leukemias, and there was no reason to believe that RadLV might be capable of acting in the same way.

Another interesting feature of the LTR is the presence of a 43 bp long duplicated sequence, containing a consensus sequence which has the characteristics of enhancer of RNA synthesis. Such sequences can exert a remote action on genes that are located thousands of bases away, and in either orientation. Proviral integration in a more or less large domain encompassing an oncogene may thus lead to tumor formation, as it has been shown not only in some avian or murine retrovirus-induced leukemias, but also in mammary tumor virus-induced cancers. Tumorigenesis due to retroviral insertion adjacent to or in the vicinity of a cellular gene has been called "insertional mutation oncogenesis".

2.1.3. A search for insertional mutagenesis by RadLV/VL3(T+L+).

For such studies, RadLV/VL3(T+L+) was used to induce thymic lymphomas in the rat rather than in the mouse, which allowed us to avoid any interference between the phenomena to be studied and those that could be attributable to the numerous endogenous mouse proviruses. Indeed, there is but a low sequence homology between mouse and rat retroviruses.

Since retroviral insertion had been described in a domain encompassing the myc oncogene of retrovirus-induced chick, mouse or rat tumors, this gene seemed to be a good candidate to initiate these studies. Out of 30 RadLV/VL3(T+L+) tumors investigated, 4 presented a modified myc domain as shown by restriction enzyme analysis. We could show that the modifications resulted from the insertion of retroviral sequences (either a complete or a partial genome) in this domain. We did not find evidence for this in the 26 other tumors, which could be supposed to have been induced by proviral insertion in the vicinity of another oncogene or by another, yet undefined mechanism.

In a next step, we designed experiments in order to investigate if the observed insertional mutagenesis could be considered as causative of cell proliferation rather than as an iniating step. Rat embryo fibroblasts were infected with RadLV and their DNA was then submitted to restriction enzyme analysis in order to examine the integration pattern of the acquired proviruses. As could be expected, the integration proved to have occurred completely at random. However, further cultivation of the

infected cultures resulted in the selective growth of cell clones, of which most displayed provirus at a given, although yet unidentified location. The same phenomenon was observed in the case of RadLV-induced mouse thymic lymphomas, which also underwent clonal growth in association with provirus in a given, though also unknown domain. From those results, we concluded that insertional mutation might not be a prerequisite for RadLV-induced lymphomagenesis. However, it may confer the cells a growth advantage in vitro and occasionally in vivo, constituting perhaps in this case a facilitating factor for the growth of some tumors.

2.1.4. A search for novel proviruses in radiation-induced thymic lymphomas.

Knowing the nucleotide sequence of RadLV/VL3(T+L+), we could easily chose the combination of a restriction enzyme and of an adequate probe to test for the acquisition of RadLV-like proviruses in radiation-induced mouse thymic lymphomas. The enzyme used was KpnI or Asp718, and the probe pEc-B4, which is homologous to ecotropic-specific sequences in the gp70 region of the RadLV-like proviral genomes.

RadLV-like proviruses were shown to have been acquired clonally in one third of the investigated lymphomas. The integration domain has yet not been identified, except in a single tumor were it turned out to be mlvi-1. Mlvi-1 is a presumed oncogene which had been reported as being activated by insertional mutation in a number of retrovirus-induced mouse or rat tumors. This was the first evidence that radiation thymic lymphomagenesis may proceed by this mechanism, albeit only occasionally. We thus wondered about the significance of the apparent absence of novel provirus in the majority of the tumors. It had been previously reported in the literature that RadLV-like retroviral particles, although most often not detectable in primary tumors, could frequently be observed in cultivated lymphoma cells. Therefore, radiation-induced tumors were submitted to in vitro cultivation or to in vivo transplantation and subsequently analysed for the presence of novel proviruses. These were found in the majority of the cases but, as in the case of most provirus-positive radiation tumors, we could not yet demonstrate their presence in a specific domain of the DNA. This situation was similar to that observed in the case of RadLV-induced lymphomas or in that of in vitro RadLV-infected fibroblasts, with respect to the existence in most of the tumors of a minor cell population that could undergo preferential growth in association with the presence of novel provirus. So, we concluded that RadLV could hardly still be regarded as an initiating factor for radiation-induced lymphomagenesis but that it could however be considered as a facilitating, although dispensable agent for tumor growth.

2.1.5. Discussion.

The leukemogenic component of the RadLV complex is a recombinant retrovirus, generated from host endogenous retroviruses that had been presumably driven to expression by radiation. Whether induced straightforwards by irradiation or indirectly in non-irradiated thymuses implanted in irradiated hosts, one third of the radiation-induced mouse thymic lymphomas display the presence, in a large proportion or in the majority of their cells, of an acquired RadLV-related provirus. They

could not be shown to be inserted in a common domain of the tumour cell DNA. In one single case, the integration had occurred in the mlvi-1 domain, a common proviral integration site in many of the the Moloney leukemia provirus induced rat thymic lymphomas. The apparently provirus-negative tumours contained a minor cell population that has an in vivo or in vitro growth advantage in relation with the presence of a novel provirus. We thus consider that, although it might play a facilitating role in the growth of a certain proportion (one third) of the radiation-induced thymic lymphomas, RadLV might not be the etiological agent.

2.2. Ras gene mutations in radiation-induced mouse thymic lymphomas.

2.2.1. Introduction.

Since the hypothesis of a viral etiology of radiation-induced thymic lymphomagenesis was no longer tenable, we reoriented our experimental work towards a search for other possible causes. It had been known for several years that mutationally activated ras genes are often associated with a variety of human or animal malignancies. Activated ras genes are presently considered as the primary cause of the development of many of those malignancies. A high proportion of radiation-induced mouse thymic lymphomas had been shown to contain an activated K-ras , or less frequently N-ras, mutated at codon 12. However, these studies involved only a very limited number of tumors, and the report that no other mutation than a GGT to GAT transition was found in our eyes quite intriguing. Indeed, we would not expect radiation to induce exclusively this unique nucleotide substitution. Moreover, nucleotide substitutions were searched for exclusively in ras genes that had been selected by transfection of cultured fibroblasts. Such in vitro transforming genes might actually not represent at all the bulk of the ras gene population in the tumor itself.

Therefore, we used the polymerase chain reaction (PCR) to amplify selectively from total thymic lymphoma DNA a ras gene segment (exon 1 + intron 1.2 + most of exon 2) encompassing codons 12, 13 and 61, reported as those that are critically mutated in relation with the acquisition of in vitro transforming activity. Thus, no bias was introduced by modifying experimentally the original proportion of the various ras alleles possibly present in the tumor. Subsequently, we also avoided the classical bias consisting in searching exclusively for predetermined mutations by molecular hybridization with a corresponding oligonucleotide probe, by determining the entire nucleotide sequence of the amplified ras gene segment. In such a way, any DNA alteration must be made visible.

Straightforward sequencing of PCR products, even from individual tumor DNA, cannot yield readable results due to the simultaneous presence of normal and of somatically mutated DNA and, as found later, to the coexistence of H-ras genes bearing different mutations. Sequencing was thus performed on a series of molecular clones from each PCR reaction.

2.2.2. Results.

The results of the sequencing experiments performed on the PCR-amplified H-ras segment are summarised in Table I.

1) Every investigated tumour was characterised by the coexistence of H-ras genes that could be distinguished from each other by one or several point mutations.

2) The observed mutations were not introduced by a PCR artefact. Among 7 H-ras clones derived from the PCR products of 2 control tissues (thymus and liver), only 2 were found to display a single -and identical- mutation involving the third base of a codon. This might represent an actual in vivo situation and not necessarily an artefact, since the degeneracy of the genetic code is compatible with the occurrence of such mutations.

3) Individual tumours are constituted by an heterogeneous cell population with respect as attested to a variety of mutated H-ras genes. This apparent lack of clonality is however limited. Two of the investigated tumours (48/1/1 and 48/1/11) yielded 2 indistinguishable mutated H-ras genes.

4) A same H-ras molecule often displays several mutations, which may be located in the exons, in the intron, or in both. Mutations in the exons often, but not always result in an amino-acid substitution.

5) Transforming mutations, considered as involved in carcinogenic processes (substitution of amino-acid 12, 13 or 61) were not observed.

6) Among 29 observed mutations, 25 were transitions (purine-purine or pyrimidine-pyrimidine substitutions) and 4 were tranversions (purine-pyrimidine substitution or vice-versa).

2.2.3. Discussion.

The large diversity of H-ras point mutations in the radiation-induced mouse thymic lymphomas, the lack of clonality of these tumours in this respect, the absence of transforming mutations in this investigated proto-oncogene, and the occasional absence of amino-acid substitutions resulting from the observed mutations, lead us to conclude that H-ras mutations are very probably not involved in the leukemogenic process. We rather postulate that the leukemic cells are hyper-mutable. Moreover, the fact that a nonnegligible proportion of the tumour cells may contain identical multiple mutations on the H-ras gene suggests that the hyper-mutability is an early event in the carcinogenic process. It seems plausible that N-ras and/or K-ras will be found to undergo similar alterations, but in addition to transforming mutations such as those described formerly in the literature. Experiments are being performed to test this prediction, as well as to investigate the possible hyper-mutability of other, non ras-related genes in the leukemic cells.

An alternative interpretation of our findings is that the variety of H-ras mutations in the radiation-induced thymic lymphomas reflects permanent DNA alterations that were caused during the irradiation. In such case, one might expect to find similar alteration in

non-tumorous tissues of the leukemic mice. Experiments are being performed to test this hypothesis. If this proved to be correct, the implications would be that measuring the number of such alterations in circulating blood lymphocytes might allow to assess radiation damage.

TABLE I. Mutations in molecular clones from PCR-amplified H-*ras* (exon1 - intron1.2 - exon2) of radiation-induced thymic lymphomas and of control tissues.

Tissue or tumour	Clone N°	Exons 1 and 2			Intron 1.2	
		codon	mutation	substitut.	position	mutation
48/1/1	1	-	-	-	-	-
	2	68	CGG-CTG	Arg-Leu	-	-
	3	60	GGT-GTT	Gly-Cys	-	-
	4,5	16	AAG-AGG	Lys-Ser	92	G-A
		50	ACA-ACG	-		
	6	18	GCC-ACC	Ala-Thr	-	-
		23	CTG-CCG	Leu-Pro		
48/1/8	1	47	GAT-GGT	Asp-Gly	-	-
	2	-	-	-	-	-
48/1/10	1	-	-	-	-	-
	2	39	TCC-TCT	-	-	-
		44	GTG-GTA	-		
		50	ACA-ACG	-		
		52	CTA-TTA	-		
		59	GCA-ACA	Ala-Thr		
		71	TAC-CAC	Tyr-His		
	3	56	TTA-TTG	-	58	A-T
		57	GAC-GTC	Asp-Val	94	T-C
48/1/11	1		-	-	36	C-T
					200	T-C
	2	38	GAC-GGC	Asp-Gly	190	T-C
		73	CGC-CGT	-		
	3,4	41	CGG-CAG	Arg-Gln	185	C-T
48/2/4	1,4	35	ACT-ACC	-	114	G-A
	2	-	-	-	-	-
	5,6	-	-	-	116	A-0
	3	81	GTA-GTG	-	-	-
	7	18	GCC-GCT	-	-	-
	8	18	GCC-GTC	Ala-Val	-	-
thymus	4 clones	-	-	-	-	-
liver	1 clone	-	-	-	-	-
	2 clones	51	TGT-TGC	-	-	-

2.3. Cellular studies.

In vivo and in vitro studies were aimed to understanding mechanisms involved in leukemogenic transformation. They should also apply to indirect leukemogenesis, i.e. transformation of a nonirradiated thymic graft in an irradiated host.

Thymic lymphomas were induced with the classical irradiation protocol (4 x 1.75 Gy) in C57BL mice. The yield was close to 100 % and the latency approximately 120 days after the last irradiation. The contribution of growth factors IL-2, IL-1 and IL-6 was evaluated by giving cyclosporin A (i.p.), or indomethacin (per os). Neither drug altered the course of the disease, suggesting that these factors were not critical for lymphomagenesis.

Tumors were also induced in NFS mice, which do not harbour ecotropic provirus susceptible to yield leukemogenic retroviruses upon recombination with a xenotropic provirus. Indirect lymphomas were obtained in thymus grafted to irradiated thymectomised C57BL or NFS recipients. Moreover, we obtained one lymphoma in an irradiated nude mouse given a thymic graft after the irradiation.

Phenotype studies were not contributory. However, we recently obtained several cell lines from indirect tomors and in four such cases, they were negative for Thy-1, Lyt-2, L3T4 and Ig.

In situ hybridisation revealed low level expression of IL-1, IL-6 and IGF-1 and this was not higher in lymphomas than in control thymus. The expression of IGF-2 was in contrast increased in 11 out of 11 primary lymphomas as compared to control thymus.

Activation mechanisms in tumor cells were investigated by looking at receptors for neurotransmitters and hormones coupled to adenylate cyclase. Lymphomas (induced by radiation or by RadLV) expressed at least one such receptor, beta-adrenergic or VIP-ergic. Receptors for VIP are remarkable since they are of the "helodermin-prefering" type.

In conclusion, our cellular and biochemical studies have documented the production of IGF-2 by radiation-induced lymphomas. IL-1, IL-6 or IL-2 do not play a critical role in lymphomagenesis. Lymphoma cells express receptors for hormones or neurotransmitters coupled to adenylate cyclase.

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V. Publications:

J. Merregaert, V.Erfle, M.Janowski, L.Michiels & A.Schön: Cellular oncogenes in viral and radiation-induced osteosarcomas. Int.J.Radiat.Biol. 49, 544-545, 1986.

J.Boniver & M.Janowski, guest editors: Symposium on Radiation Carcinogenesis (Molecular and Cellular Aspects). Special Issue of Leukemia Research, vol. 10, Pergamon Press, 1986, 234 pages.

J.Merregaert, L.Michiels, E.Vander Rauwelaert, M.Lommel, R.Gol-Winkler & M.Janowski: Oncogene involvement in radiation- and virus-induced mouse osteosarcomas. Leukemia Res.10, 915-921, 1986.

M.Janowski, J.Merregaert & E.P.Reddy: Retroviruses in radiation-induced lymphomas. Leukemia Res.10, 833-842, 1986.

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R. Hooghe, M. Janowski, R. Greimers & N. Schaaf-Lafontaine : Radiation-induced lymphoma developing during Cyclosporine A treatment. Transplantation, 48, 342-344, 1989.

M. Janowski, R. Cox & P.G. Strauss: The molecular biology of radiation-induced carcinogenesis: Thymic lymphoma, myeloid leukaemia, and osteosarcoma. Int.J.Radiat.Biol., in press.

Title of the project no.: 2

Molecular biology of radiation-induced osteosarcomagenesis. Role of oncogenes and viruses.

Head(s) of project:

B. Borremans

Scientific staff:

B.Borremans
M. Janowski

I. Objectives of the project:

To identify and characterise genes submitted to critical alterations in relation with radiation induced osteosarcomagenesis.

II. Objectives for the reporting period:

Searching for reorganisation of proto-oncogenes.

III. Progress achieved:

1. Methodology

Classical restriction enzyme analysis of DNA with specific probes for molecular hybridization.

2. Results

In the frame of project n° 1, we obtained indications that the presumed proto-oncogene Mlvi-1 seems sometimes to be involved in radiation leukemia virus-induced leukemogenesis of rats. On the other hand, our colleagues of the INSERM Unit n° 117 (Bordeaux, France) performed the molecular cloning of a gene that could be rearranged in radiation-induced leukemogenesis of C57BL mice. Our colleagues of the GSF (Neuherberg, FRG) provided us with a series of DNAs from radiation-induced osteosarcomas of Balb/c mice. We tested these DNAs for rearrangements of the gene that was cloned in Bordeaux, and found that it was frequently and considerably amplified. It turned out that this amplifications occurred in the tumors that already had been shown to display concommitant amplification of the proto-oncogene c-myc and of the presumed oncogene Mlvi-1. This result contributed to the identification of the gene studied in Bordeaux as Mlvi-1, and simultaneously confirmed the observations made in Neuherberg.

3. Discussion.

Radiation-induced osteosarcomas of the Balb/c mouse frequently display concommitant amplification of the proto-oncogene c-myc and of the presumed proto-oncogene Mlvi-1. It has been presently known that this phenomenon is due to the appearance of multiple minute chromosomes containing both c-myc and Mlvi-1. However, the contribution of these minute chromosomes to the osteosarcomagenetic process is still unknown.

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V. Publications:

J. Merregaert, V.Erfle, M.Janowski, L.Michiels & A.Schön: Cellular oncogenes in viral and radiation-induced osteosarcomas. Int.J.Radiat.Biol. 49, 544-545, 1986.

J.Boniver & M.Janowski, guest editors: Symposium on Radiation Carcinogenesis (Molecular and Cellular Aspects). Special Issue of Leukemia Research, vol. 10, Pergamon Press, 1986, 234 pages.

J.Merregaert, L.Michiels, E.Vander Rauwelaert, M.Lommel, R.Gol-Winkler & M.Janowski: Oncogene involvement in radiation- and virus-induced mouse osteosarcomas. Leukemia Res.10, 915-921, 1986.

A.Schön, L.Michiels, M.Janowski, J.Merregaert & V.Erfle: Expression of proto-oncogenes in murine osteosarcomas. Int.J.Cancer 38, 67-74, 1986.

J.Merregaert, M.Janowski & E.P.Reddy: Nucleotide sequence of a radiation leukemia virus genome. Virology 158, 88-102, 1987.

M. Janowski, B. Borremans, R. Hooghe, J. Merregaert, P. Reddy, J. Boniver, M.P. Defresne : Search for genes involved in murine thymic lymphomagenesis. in : Radiation Research, E.M. Fielden, J.F. Fowler, J.H. Hendry and D. Scott, Editors, Taylor and Francis, London, 1987, pp. 482-487.

H.Baylac-Kalabokias, T.Astier-Gin, B.Borremans, E.Legrand, R.Hooghe, M.P.Houben-Defresne, M.Janowski, J.F.Duplan & B.Guillemain: Evidence of recombinant ecotropic provirus integration in thymic lymphomas induced by direct or indirect radiation effects. Leukemia Res.13, 131-143, 1989.

M. Janowski, R. Cox & P.G. Strauss: The molecular biology of radiation-induced carcinogenesis: Thymic lymphoma, myeloid leukaemia, and osteosarcoma. Int.J.Radiat.Biol., in press.

RADIATION PROTECTION PROGRAMME

Progress Report

1989

Contractor: Contract no.: BI6-D-319-F

Inc. Agency for Research on Cancer
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Title of the research contract:

Survey of childhood leukemia incidence in Europe, with particular reference to the Chernobyl accident.

List of projects:

Survey of childhood leukemia incidence in Europe, with particular reference to the Chernobyl accident.

EUROPEAN CHILDHOOD LEUKAEMIA/LYMPHOMA INCIDENCE STUDY

Progress Report

A meeting of collaborators was held, following the annual meeting of the International Association of Cancer Registries, in Maastricht, Netherlands, on 21 September, with Dr Chadwick, Radiation Protection Programme of the CEC in attendance. A report of the meeting is attached.

Several modifications to the system of data collection were proposed, in the light of experience of the submission of leukaemia case information from the baseline period, 1980-86. The results from most of the centres for the baseline (pre-accident) period were available, and presented to the meeting. There was a discussion on the most appropriate methods for analysing the data for underlying time trends. Registries will be expected to furnish their 1987 data (updating the entire period 1980-87 at the same time) by the end of 1989. A more formal presentation of the leukaemia incidence data was scheduled for the first half of 1990.

The recommendations from the committee of experts convened earlier in the year by the CEC Radiation Protection Programme were discussed. It was not felt feasible to extend the study to all childhood cancers. However, it was agreed that data on childhood lymphomas should also be collected and analysed, primarily because of the difficulties in distinguishing certain leukaemias from non-Hodgkin lymphomas, rather than any likely change in incidence as a result of radiation exposure.

A revised protocol of the study was prepared, taking account of these modifications, and has been circulated to all participants in November (IARC Internal Report 89/002 Rev. 1 attached).

Several contacts have been made in the past with UNSCEAR. It is proposed to use the computer programme developed by UNSCEAR to refine the Chernobyl exposure estimation down from the rather large geographical areas used in the 1988 UNSCEAR report to areas of an appropriate population size for epidemiological

analyses of childhood leukaemia (1-2 millions). These exposure estimates will be calculated over the next 6 months, and before any analysis takes place of leukaemia incidence data from after 1986.

Continuation of study

As described above, the first formal statistical analyses of childhood leukaemia risk in relation to estimated radiation exposure will take place in 1990, when data from 1980-1987 will have been received. It is proposed that a report will be prepared for possible publication during 1990. A publications policy has been agreed amongst the collaborators, and incorporated into the protocol. The study should continue for at least 4 additional years, so that data for a minimum of 5 years post-Chernobyl are available. Detailed data analyses will take place each year. Meetings of participants are anticipated at approximately two-yearly intervals, in general to coincide with the annual meeting of the International Association of Cancer Registries.

TABLE 1

Participating centres	Approx. 1985 Childhood population (mill.)
Austria (National data on childhood leukaemia)	1.3
Czechoslovakia (Registries of Slovakia, Moravia, Bohemia)	3.8
Denmark	1.0
Finland	0.9
France (2 Paediatric registries: Lorraine, PACA-Corse) (1 Haematology registry: Côte d'Or) (2 General registries: Bas-Rhin, Isère)	approx. 1.6
German Democratic Republic	3.1
Federal Republic of Germany	9.6
Hungary	2.4
Italy (Paediatric registry - Torino) (General registries: Varese, Genoa, Tuscany, Latina, Ragusa, Piemonte)	1.3
Netherlands (Childhood Leukaemia Study Group)	2.5
Norway	0.8
Poland	9.4[1]
Sweden	1.5
Switzerland (Registries of Basel, Geneva, Neufchâtel, St. Gallen, Vaud and Zürich)	0.5
UK: England & Wales	11.1
Scotland	1.3
USSR: Estonia	0.3
Lithuania	0.8
Yugoslavia: Slovenia	0.5

[1] Quality of registration varies by province, and data from a selected number will be retained.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor:

Contract no.: BI6-D-086-DK

University of Aarhus
Ndr. Ringgade 1
DK-8000 Aarhus C

Head(s) of research team(s) [name(s) and address(es)]:

Dr. N.O. Kjeldgaard
Dept. Molecular Biol. & Plant Phy.
University of Aarhus
C.F. Møllers Allé 130
DK-8000 Aarhus C

Telephone number: 06-125177

Title of the research contract:

Characterization of somatic mutations during radiation induced osteosarcomagenesis.

List of projects:

1. Characterization of somatic mutations during radiation induced osteosarcomagenesis.

Title of the project no.: 1

Characterization of somatic mutations during radiation induced osteosarcomagenesis.

Head(s) of project:

N. O. Kjeldgaard and F. S. Pedersen

Scientific staff:

M. Duch, E. C. Jørgensen, P. Jørgensen, N. O. Kjeldgaard, K. Lidegaard, J. Lovmand, S. Lovmand, N. Pallisgaard, F. S. Pedersen, L. Pedersen.

I. Objectives of the project:

1) To identify genetic changes in proviral genes and other cellular genes associated with integrated proviruses occuring during the development of bone tumours.

2) To determine the functional role of these mutations and to evaluate their role in osteosarcomagenesis.

II. Objectives for the reporting period:

1) To isolate and characterise additional viruses from bone tumours.

2) To perform detailled transcriptional analyses of the isolated viruses.

3) To analyse the cellular genes surrounding integrated proviruses in bone tumours.

III. Progress achieved:

Methodology

All our work during the period 1985 through 1989 has employed murine retroviruses as tools for the study of radiation induced carcinogenesis. All viruses are available as molecular clones, and standard techniques of molecular biology are used throughout the work.

Results and discussion

Isolation and characterisation of viruses. One section of the research concerns the structure and function of murine leukemia viruses associated with bone tumours and with lymphoid tumours. This part aims at identifying genetic changes specifically associated with the development of bone tumours. As well viruses from radiation-induced as from non-radiation induced tumours are included in the analysis. The viruses are characterized with respect to their pathogenic properties in laboratory mouse strains and their genomic structures have been analyzed by partial or complete nucleotide sequence analysis. A hypervariable region has been identified in the part of U3 associated with transcriptional enhancement. The structure of this region is important for the activation and expression of the viruses in different tissues. The biological properties of the viruses and the structures of their repeat modules are summarised in Table 1 and Figure 1.

Virus strain	Malignant lymphoma	Osteopetrosis	Osteoma
p7D	9/10 (365 d)	5/10 (320 d)	1/10
pN20-7	8/9 (333 d)	8/9 (376 d)	3/9
lambda OTS-25	12/19 (475 d)	2/19 (601 d)	0/19
lambda OTS-72	4/8 (417 d)	2/8 (396 d)	0/9
Mock	3/9 (258 d)	0/9	0/9

Table 1. Pathogenicity in NMRI mice of murine leukemia viruses derived from BALB/c mice. Molecular clones: p7D (Horowitz and Risser, J. Virol. 56:798-806, 1985) harbours the endogenous ecotropic provirus of the BALB/c mouse, and pN20-7 (Rassart et al., J. Virol, 39:162-171, 1981) harbours an exogenous derivative of the endogenous virus. The clones lambdaOTS-25 (Strauss et al., 1988) and lambdaOTS-72 (L. Pedersen, Thesis, Aa. U., 1987, manuscript in preparation) represent somatically acquired proviruses isolated from two independent radiation-induced osteosarcomas of the BALB/c mouse strain. Mean latency periods (days) are given.

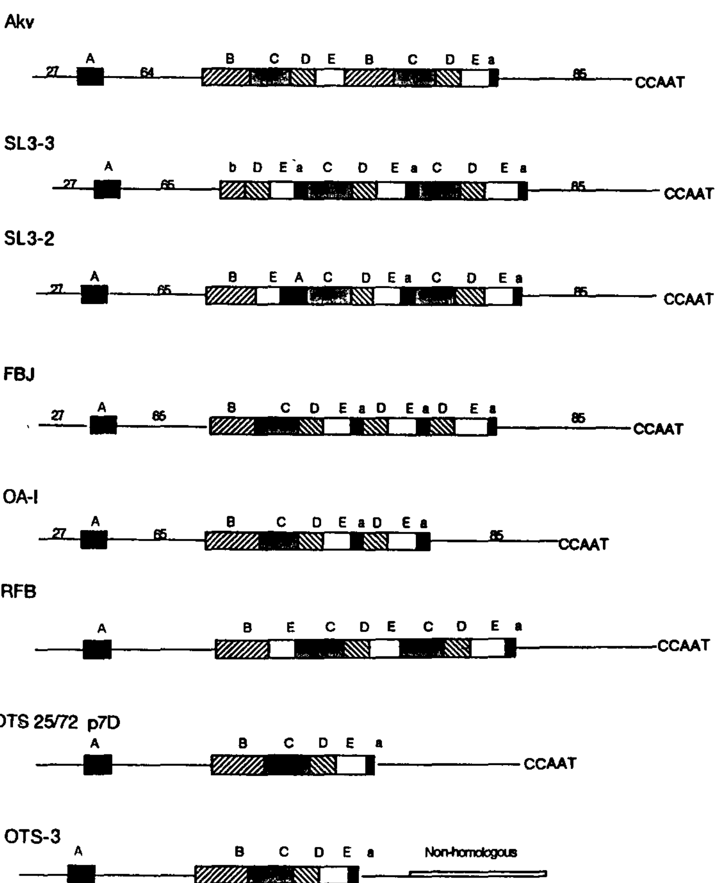


Figure 1. Modular arrays of the repeat structures of selected MuLV U3 regions. Akv is the endogenous ecotropic virus of the AKR strain. SL3-3 and SL3-2 (Dai et al. 1990) are lymphoma derived AKR viruses. FBJ, OA-I (Leib-Mösch et al. 1986), and RFB (manuscript in preparation) are bone tumour derived viruses. OTS-3 represents a non-infectious somatically acquired provirus from a radiation-induced osteosarcoma of a BALB/c mouse (manuscript in preparation). The two LTRs of this virus differ; the downstream U3 region is shown. The remaining viruses are described in Table 1. The sequences of the modules and the point mutations relative to the Akv sequence are given in Pedersen et al. (1987). Modules indicated by lowercase letters contain only part of the normal module sequence. The U3 sequences outside the modules are identical or nearly identical to the Akv sequences.

Retrovirus derived vectors. Our studies of provirus activation and expression are carried out with defective proviruses to avoid uncontrolled spread of viruses during the experiment and to avoid the mutations that may accompany virus replication. To allow easy measurement of virus expression levels we use retroviral vectors with a prokaryotic reporter gene, either the gene encoding chloramphenicol acetyltransferase (CAT) or the neo gene encoding neomycin phosphotransferase II (NPTII). We have constructed a number of such vectors that allow amplification of the DNA in prokaryotic vectors before introduction into mammalian cells in culture. The vectors are of two types. The structure of the expression vectors that exploit signals in the viral DNA for control of transcription is summarised in Figure 2. Vectors of this type are used for measurement of the strength of the transcriptional signals in variant and mutated U3 regions in different cells in culture. This type of assay measures gene expression driven by unintegrated DNA. The transmission-expression vectors (Figure 3) are designed to allow one cycle of replication of the virus vector after introduction into a helper cell-line that provides all necessary proteins. This type of vectors allows determination of gene expression driven by a vector provirus integrated into chromosomal DNA of the host. Both types of vectors are based on the same prototype murine leukemia virus, Akv, and allow easy exchange of Akv U3 sequences with U3 sequences from other viruses (Figure 2 and Figure 3).

Identification of transcription signals in unintegrated vectors. This part of our work is directed towards the identification of nucleotide sequence elements in the U3 region that regulate transcription by binding of specific host-proteins. Such proteins may be general or differentiation-specific transcription factors or may be factors that are activated in cells in response to environmental agents. Our strategy for this analysis has been to perform a detailed genetic analysis of a prototype U3 region (from Akv) in one cell line (NIH 3T3) and subsequently to use the results from this analysis as the basis for studies of other U3 regions and other cell lines. A large number of U3 deletions has been introduced into the pAkv6-vector and their effect on expression analyzed. The effect of upstream deletions extending into U3 is summarised in Fig. 4. The results show a successive reduction in expression activity as a result of successive deletions and point to critical functions of specific sequences in the repeat module region. Additional studies using site-specific mutagenesis and purified protein fractions (data not shown, H. S. Olsen et al., manuscript submitted) have determined that the critical sequences mediate their transcriptional function by serving as a target for the binding of Nuclear Factor I proteins, a group of sequence specific DNA binding proteins in the host cell.

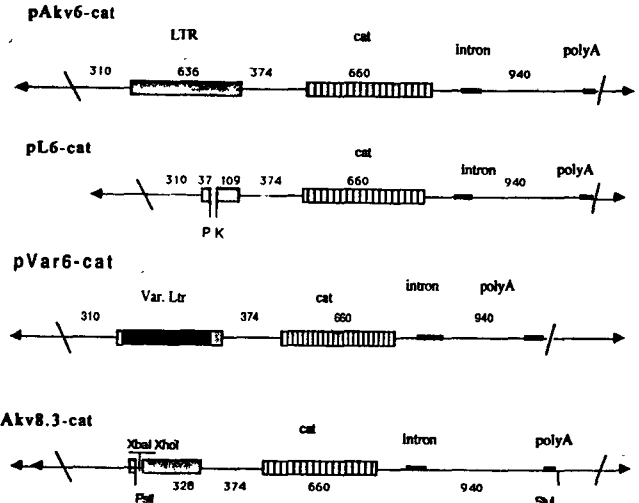


Figure 2. Genomic structure of the expression vector pAkv6-cat and derived vectors (Lovmand et al., manuscript submitted, Pedersen et al., 1987, Kjeldgaard et al., 1989, Paludan et al., 1989a, Dai et al., 1990). The vectors contain an intron and the polyA signal from SV40. The numbers indicate the length of the fragments in base pairs. The unique restriction sites PstI (P) and KpnI (K) allow insertion of appropriate variant U3 regions as shown (pVar6-cat). The pAkv8.3-cat vector and the related pAkv8.2-cat and pAkv8.1-cat vectors (not shown) allow insertion of various fragments in a deleted U3 region or in a position downstream of the transcription unit.

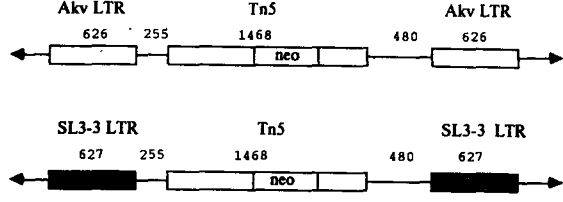


Figure 3. Structure of transmission-expression vectors (Jensen et al., 1986, Paludan et al., 1989a, 1989b). The vectors are shown as integrated vector proviruses in a mammalian host cell. The arrows denote host cell DNA. A simple construction scheme allows U3 replacement; A vector with the OA-I LTR has also been generated (not shown).

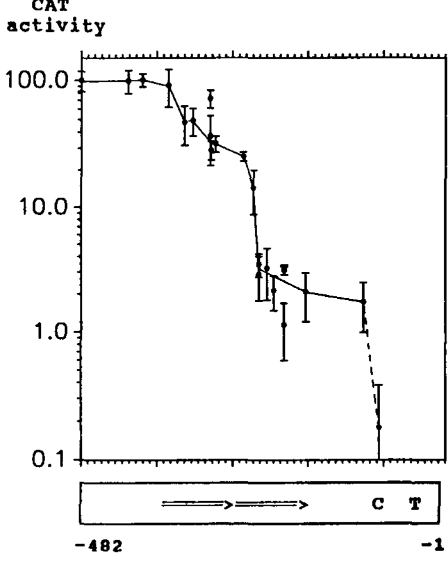


Figure 4. Effect of upstream deletions in U3 of Akv upon transient expression activity. Plasmid DNAs of derivatives of pAkv6-cat (Figure 1) with upstream deletions in U3 were introduced into NIH 3T3 cells and the level of vector gene expression was determined by enzymatic measurements of the reporter gene product, CAT. The activity of undeleted pAKV6-cat is arbitrarily set to 100 and relative activity levels of the deleted pasmids marked on a logarithmic scale. The extent of the deletions is mapped on the complete U3 region from base pair -1 to base pair -482. The arrows mark the two tandem repeats (modules B, C, D, E in Fig. 1) and C and T the CCAAT and TATA boxes, respectively.

In a separate series of experiments we have analysed the role of tandem repeat sequences in gene expression of murine retroviruses. As shown in Figure 1, tandem repeats are widely occuring in the group of viruses under study, and both the mechanisms behind repeat formation and the role of the repetitions for provirus activation and expression are central in our work. For these studies our model system has again been the Akv U3 in NIH 3T3 cells. The vectors pAkv8.1-cat, pAkv8.2-cat, and pAkv8.3-cat have been used for insertion of fragments of the Akv U3 region either in an upstream position or in a downstream position. Figure 5 shows a comparison of the activities of pairwise homologous constructs, differing only in their having one or two copies of the tandem repeat sequence. The results indicate a complex pattern of cooperativity between repeat sequences and U3 sequences outside the repeats. Plasmids with two repeat copies show high activities in all cases, whereas the precise sequence environment seems to have a marked effect upon the homologous constructs with only one repeat copy. Thus, the repetitions do not only seem to increase the expression level, but may also serve to make the repeat units function more independently of flanking DNA.
Less detailed expression studies (data not shown, Dai et al. (1990), Kjeldgaard et al., (1989), L. Pedersen et al., manuscripts in preparation) have been carried out in a number of cell lines for Akv and the variant LTRs from OA-I, RFB, SL3-2, SL3-3 and other viruses. In our recent work, an effect

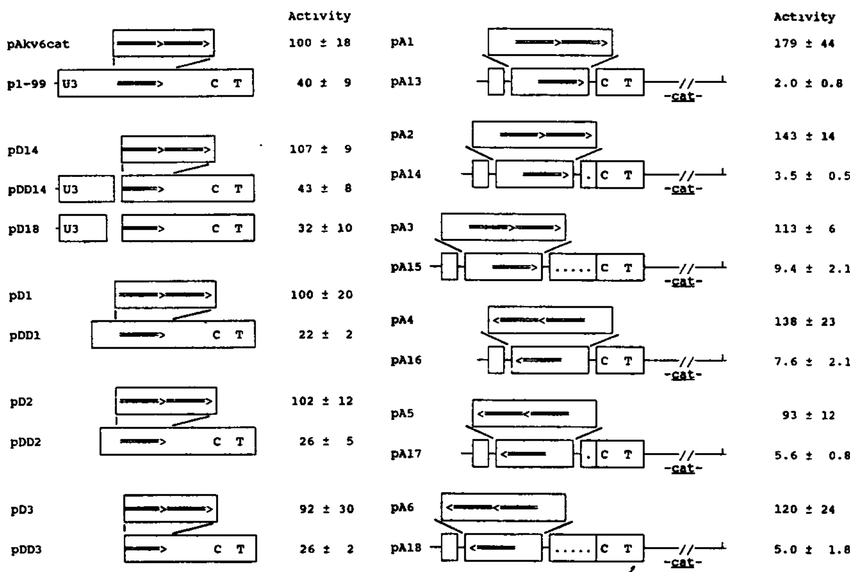


Figure 5: Expression activities in presence of one or two tandem repeat units (S. Lovmand et al., manuscript submitted). Deletion derivatives of pAkv6-cat (left panel) and derivatives of pAkv8.1-cat, pAkv8.2-cat, and pAkv8.3-cat were used for transfection of NIH 3T3 cells and the gene expression activities determined as in Fig. 4. The boxes represent contiguous Akv U3 sequences and the lines non-U3 sequences.

of the oncoprotein and stress-protein, Fos, on the expression from the Akv and OA-I U3 regions in osteogenic cells has been detected by co-transfection of fos-expression vectors (unpublished, data not shown). Such mechanisms may also play a role in induction of retroviruses in bone tissues by radiation. Another part of our present work studies the function of E modules of slightly different structure (Figure 1) in U3 of OA-I, FBJ, and Akv viruses. A protein binding to these sequences has been purified from NIH 3T3 cells and its function in transcriptional regulation is being elucidated.

Regulation of transcription of integrated proviral vectors. In this part of our work we use the transmission-expression vectors described in Figure 3 to study the expression from individual vector proviruses integrated at different sites in the host chromosomes (Paludan et al. 1989a, 1989b, Jensen et al., 1986) using cell clones with only one provirus of apparently intact structure as detected by sequence analysis

after amplification by the polymerase chain reaction. The cell clones were obtained without selection for expression of the introduced neo gene. Three regulatory principles may be analysed by this approach: the regulatory DNA elements of the U3 region, the regulatory factors in the host cell that may interact with the DNA elements, and the regulatory role of chromosomal region of integration. The level of provirus-expression may be measured under steady-state conditions as in Paludan et al. 1989a, 1989b, or their response to environmental agents may be studied. Figure 6 shows the results of steady-state measurements of provirus expression during continuous growth of the host cell, in this case the lymphoma cell line L691. Two different U3 regions (from Akv and SL3-3, Figure 3) with different transcriptional strength

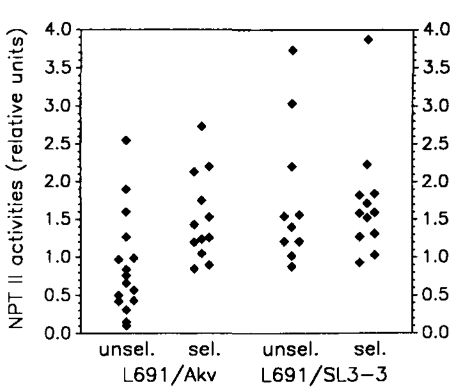


Figure 6. Expression activities of integrated Akv and SL3-3 proviruses (Paludan et al., 1989a, 1989b). The expression activities were determined from single vector proviruses in individual unselected L691 cell clones (unsel.) as the enzymatic NPT II activity. The values are given relative to the activity of an arbitrary standard extract. The columns marked with sel. represent the NPT II activity in cell clones derived from infected populations selected for resistance to the antibiotic G418.

in the host cell were employed. Integrated proviruses of identical structures show wide differences in expression levels (unselected L691/Akv and L691/SL3-3 values, Figure 6), to some extent offsetting the inherent differences in U3 strengths, as detected in unintegrated DNA. According to our interpretation, the inherent U3 strengths may also determine the lowest expression values for each type of vector, and the higher values may represent positive chromosomal influence. Figure 6 also shows the effect upon expression levels of selection for the neo reporter gene expression by growth in the antibiotic G418. The effects of NPT II expression levels upon cell growth and survival in the presence of G418 are further documented by the results of a growth-rate analysis presented in Figure 7. These results demonstrate that the full range of variation in expression levels among individual transferred genes may not be observed when antibiotics-selected cell material is used, as is commonly the case in this type of gene transfer experiments.

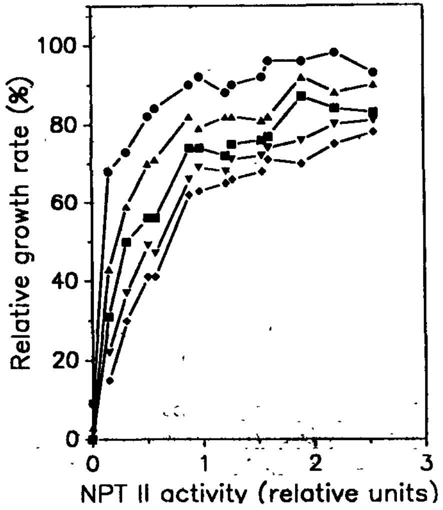


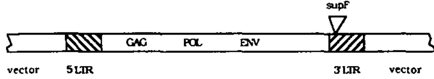
Figure 7. Relative growth rates as functions of NPT II activity in the presence of various G418 concentrations (Paludan et al., 1989b). The exponential growth rates were determined for 13 cell clones and for uninfected L691 cells, without G418 and with five different G418 concentrations. G418 determined growth rates relative to the growth rates without G418 are shown as functions of NPT II activity. The G418 concentrations were (curves from top to bottom), 0.5 mg/ml. 0.9 mg/ml, 1.4 mg/ml, 1.8 mg/ml, and 2.2 mg/ml.

In summary, the material described in this section represents a number of cell clones, each with one vector transcription unit of identical structure inserted at different sites in the host DNA. The expression-level from the transcription unit varies about 25-fold among cell clones (for the Akv-neo proviruses), but completely inactive clones of apparently intact structure have also been isolated. The material has unique potentials for the study of the effect of environmental agents such as radiation on as well mutation rates as expression levels under various types of chromosomal influence.

Identification of oncogenic target genes of the host. Replication competent retroviruses may exert their oncogenic effect as proviruses integrated at specific sites of the host chromosome by acting as mutagens that disturb genes involved in growth control. Retroviral elements activated during radiation carcinogenesis may play a role in this manner. The group of host genes that can be oncogenetically activated by provirus insertion may also be sensitive to other kinds of mutations. To facilitate the analysis of proviral integration sites we have employed provirus tagging techniques. This approach may be particularly relevant for tumours of the bone, where only small quantities of DNA can be extracted. The tagged viruses are replication competent, but carry, in addition to the complete virus genome, a short nucleotide sequence that allow easy reisolation of integrated proviruses and their flanking host DNA in prokaryotic vectors. The *supF* tagging system is described in P. Jørgensen et al., (1988) and in Figure 8. A similar tagging system employing the *lacO* region has also been developed (data not shown). The tagged

viruses have retained their pathogenicity, although the latency period may be somewhat prolonged as shown in Figure 9 for the lymphomagenic SL3-3 virus. Using this approach, the proviral integration sites in a number of tumour DNAs have been analysed by their nucleotide sequence.

Prokaryotic vector carrying a viral genome with supF inserted in the 3 LTR



Provirus inserted in host DNA after replication in the eukaryotic cell

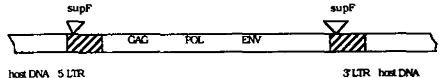


Figure 8. Tagging of the provirus with the prokaryotic supF gene (P. Jørgensen et al., 1988). A short DNA segment encoding the prokaryotic selectable marker gene, supF, was inserted at the PstI site in the downstream U3 of the provirus in an *E. coli* plasmid vector. An infectious virus stock was generated by transfection of mouse cells. In agreement with the retroviral life-cycle both U3 copies of progeny proviruses were found to contain the supF gene (lower panel).

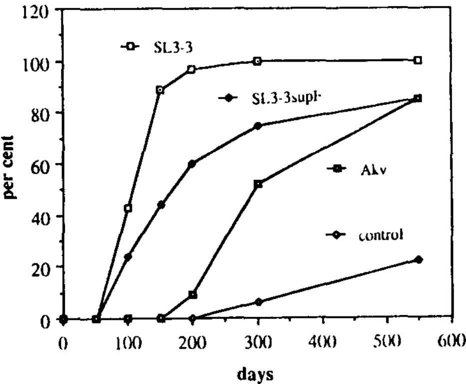


Figure 9. Pathogenicity of SL3-3, SL3-3supF, and Akv Mulvs in NMRI mice. Newborn mice were injected with medium from virus producing NIH 3T3 cultures, obtained after transfection of molecular clones. The number of mice with malignant lymphomas at death was recorded as per cent of mice inoculated at the days indicated in the graph. The total number of mice was 35 for SL3-3, 75 for SL3-3supF, 28 for Akv, and 18 for the controls.

The integration sites of somatically acquired proviruses in radiation-induced osteosarcomas of BALB/c mice have been analysed (Strauss et al. 1988, unpublished). No common integration sites have been detected. The integration sites are being further investigated to analyse for homology to known sequences and to test for expression of the sequences of the pre-integration site.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

The work is carried out in close collaboration with the research group at GSF/Neuherberg (Erfle) under the coordination of EULEP Task Group no. 1 "Radiation induced osteosarcomagenesis".

V. Publications:

Erfle,V., Schmidt,J., Leib-Mösch,C., Pedersen,F.S., & Luz,A.: Einfluss der Genomstruktur von Retroviren auf die Entwicklung von Knochentumoren. In Bericht des 16. Kongresses der Deutchen Veterinärmedizinischen Gesellschaft, pp 118-125, Verl. Parey, Berlin, Hamburg 1985.

Leib-Mösch,C., Schmidt,J., Etzerodt,M., Pedersen,F.S., Hehlmann,R. & Erfle,V.: Oncogenic retrovirus from spontaneous murine osteomas. II. Molecular cloning and genomic characterization. Virology 150, 96-105 (1986).

Jensen,N.A., Jørgensen,P., Kjeldgaard,N.O. & Pedersen,F.S.: Mammalian expression-and-transmission vector derived from Akv murine leukemia virus. Gene 41, 59-65 (1986)

Pedersen,F.S. & Etzerodt,M.: Structure of endogenous retroviruses expressed in radiation-induced and spontaneous murine bone tumours. Leukemia Res., 10, 923-930. (1986). 28.

Jørgensen,P. & Mikkelsen,T.: Lambda-PJ4A, a lambda replacement vector carrying amber mutations for cloning of EcoRI fragments. Nucl. Acids Res. 14, 9538 (1986).

Pedersen,F.S., Etzerodt,M., Lovmand,S., Dai,H.Y., Bækgaard, A.J., Sørensen,J., Jørgensen,P., Kjeldgaard,N.O., Schmidt, J., Leib-Mösch,C., Luz,A. & Erfle,V.: Transcriptional control and oncogenicity of murine leukemia viruses. In Viral Carcinogenesis, Alfred Benzon Symposium 24 (N.O. Kjeldgaard & J. Forchhammer, eds.), pp 17-35, Munksgaard, Copenhagen 1987.

Jensen,N.A., Jørgensen,P., Kjeldgaard,N.O. & Pedersen,F.S.: Expression of BPV1 late genes in cultured cells using retroviral vectors. Cancer Cells 5, 131-135 (1987).

Jørgensen,P., Mikkelsen,T., Pedersen,F.S. & Kjeldgaard,N.O.: A MuLV transmission vector system designed to permit recovery in E.coli of proviral and cellular flanking sequences. Virus

Genes 1, 221-233, (1988).

Strauss,P.G., Schmidt,J., Pedersen,L., & Erfle,V.: Amplification of endogenous proviral MuLV sequences in radiation-induced osteosarcomas. Int. J. Cancer, 41, 616-621 (1988).

Copeland,N.G., Jenkins,N.A., Nexø,B., Schultz,A.M., Rein,A., Mikkelsen,T., & Jørgensen,P.: Poorly expressed endogenous ecotropic provirus of DBA/2 mice encodes a mutant Pr65gag protein that is not myristylated. J. Virol. 62, 479-487 (1988).

Jørgensen,E.C., Kjeldgaard,N.O., Pedersen,F.S., & Jørgensen, P.: A nucleotide substitution in the gag N terminus of the endogenous DBA/2 virus prevents Pr65-gag myristylation and virus replication. J. Virol. 62 , 3217 -3223 (1988).

Kjeldgaard,N.O., Bækgaard,A.J., Dai,H.Y., Etzerodt,M., Jørgensen,P., Lovmand,S., Olsen,H.S., & Pedersen,F.S.: Transcriptional control by retroviral LTR regions. In Evolutionary Tinkering in Gene Expression. NATO Advanced Summer Institute Series (M. Grunberg-Manago ed.), pp 82-94, Plenum Publishing Corp., New York, USA, 1989.

Paludan,K., Dai,H.Y., Duch,M., Jørgensen,P., Kjeldgaard,N.O., and Pedersen,F.S.: Different relative expression from two murine leukemia virus long terminal repeats in unintegrated transfected DNA and in integrated retroviral vector proviruses. J. Virol. 63, 5201 - 5201 (1989a).

Paludan,K., Duch,M., Jørgensen,P., Kjeldgaard,N.O., and Pedersen,F.S.: Graduated resistance to G418 leads to differential selection of cultured mammalian cells expressing the neo gene. Gene 85, 423 -428 (1989b).

Pallisgaard,N., Mikkelsen,T., Pedersen,F.S., Kjeldgaard,N.O., and Jørgensen,P. The nucleotide sequence of the 3' region of the murine leukemia virus SL16c4. Nucl. Acids Res. 17, 6413 (1989).

Dai.H.Y., Etzerodt,M., Bækgaard,A.J., Lovmand,S., Jørgensen,P., Kjeldgaard,N.O., and Pedersen,F.S.: Multiple sequence elements in the U3 region of the leukemogenic murine retrovirus SL3-2 contribute to cell-dependent gene expression. Virology, in press, (1990).

Pallisgaard,N., Pedersen,F.S., Kjeldgaard,N.O., and Jørgensen,P.: Cloning of cDNAs for proteins binding to the MuLV enhancer region. In Gene Regulation, Oncogenesis, and AIDS, Portfolio Publishing Company, The Woodlands, Texas, in press (1990).

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-202-NL

Rijksuniversiteit Leiden
Stationsweg, 46
NL-2300 RA Leiden

Head(s) of research team(s) [name(s) and address(es)]:

Prof. P.H.M. Lohman
Labor. voor Stralen. & Chem. Mutag.
Rijksuniversiteit Leiden
Wassenaarseweg, 72
NL-2333 AL Leiden

Telephone number: 071-14833 Ext. 6176/6150

Title of the research contract:

Genetic and molecular characterization of stages in X-ray induced malignant transformation.

List of projects:

1. Investigation of the number and the genetic and molecular nature of the events, such as immortalization, transformation, oncogene activation, promotion, which are involved in radiation induced carcinogenesis using Syrian hamster embryo cells.

Title of the project no.:

Investigation on the number and the genetic and molecular nature of the events, such as immortalization, transformation, oncogene activation, promotion, which are involved in radiation induced carcinogenesis using Syrian hamster embryo cells.

Head(s) of project:

Dr. J.W.I.M. Simons.

Scientific staff:
Drs. A.J. de Kok, Drs. B. Bols, Drs. J. Boesen.

I. Objectives of the project:

This project aims to further define the events in malignant transformation of cultured cells. These events are immortalization, transformation and promotion. The search will be for genetic mechanisms and genes involved.

II. Objectives for the reporting period:

1. Kinetics of induction of immortalization.
2. Two-step probabilistic phenomenon in immortalization.
3. Characterization of newly immortalized cell lines.
4. Effect of tumor promotor on immortalization.
5. Effect of tumor promotor on genetic stability.
6. Expression of proto-oncogenes in successive stages of transformation of SHE cells.

III. Progress achieved:

1. Kinetics of induction of immortalization.

Methodology.
Tertiary cultures of SHE (Syrian hamster embryo) cells have been treated with either B(α)P (20 μg/ml), X-rays (300 rad) or ENU (3mM). The treated cells were split into a large number of subpopulations and cultured for 6 days in complete medium for expression of induced mutations. Subsequently each population was cultured in medium with low serum in order to select for immortalized cells, The cultures were propagated until senescence or escape from senescence was observed. This immortalization assay allows the monitoring of the growth rate and cloning efficiency (CE) of each population in the successive passages. Immortalization frequencies were determined with the P-zero method on the proportion of cultures which were mortal.

Results.
Contrary to expectation immortalization did not occur in a single step but required at least three steps: all populations which escaped normal senescence (step 1) invariably still had a low CE of about 0.1-1%. Increase of the cloning efficiency to 30 a 70% (step 2) occurred sooner or later via the sudden appearance of variant cells which replaced the original cell population. Many of these cell populations were slow growing during these two changes. In the majority of these slow growing cultures a second sudden change was observed in which fast growing cells took over (step 3). An example of the occurrence of the three steps in immortalization is shown in figure 1 (line BP-11).
It appears that all three steps are required for full immortalization as senescence still has been observed in cell lines which underwent only the first or the first and second step: six lines only underwent the first step and displayed an extended lifespan when compared with the controls but all six became ultimately senescent. Another six cell lines only underwent the first and the second step and two of them became senescent within the course of the experiment (150 days). After the third step the cell lines are remarkably similar in growth rates and behave like true established cell lines.
The frequencies of the three steps have been calculated (table 1). The frequencies of the first step have been calculated on the number of seeded cells. These frequencies are in the order of 10^{-5} a 10^{-4} which is in the order of magnitude of induced mutation frequencies at the HPRT locus in mammalian cells by these agents.
The frequencies of the second and third step have been calculated per cell per generation The overall frequency is $6.3X10^{-8}$ for the second step (increase in CE) and $4.3X10^{-8}$ for the third step (increase in growth rate). These frequencies are in the order of magnitude of spontaneous mutation frequencies.
Spontaneous immortalization has not been observed. This means that the spontaneous frequency of immortalization must be below $2.4X10^{-9}$ per cell per generation.

Discussion.
Steps in immortalization.
The findings suggest that immortalization in itself is a multistep process. At least three steps are required: 1) escape from normal

senescence, 2) increase in CE and 3) increase in growth rate. Newly immortalized cells have been reported to be non-malignant. Malignancy has been shown to correlate with growth in agar. Crawford et al (1983) demonstrated that immortal SHE cells required the ability to grow in agar with a frequency of $5.2X10^{-8}$ per cell per generation. This means that at least four steps are required for neoplastic conversion of primary SHE cells.

The results indicate that these steps are sequential and dependent on each other: as sudden increases in the CE (step 2) are never observed in normal cells the first step must be considered as a prerequisite for the occurrence of the second step. It is indicated that the second step is a prerequisite for the third step as in all the cell lines in which these three steps can be recognized (12 lines) the increase in CE precedes the increase in growth rates. The same appears to hold for step 3 and step 4 (grow in agar) because none of the newly immortalized cell lines was able to grow in agar (see section 3).

The importance of these findings are discussed in the "general discussion".

Spontaneous immortalization.

In none of the experiments spontaneous immortalization was observed which means that the rate must be below $2.4X10^{-9}$ per cell per generation. This seems in contrast to many publications which report spontaneous immortalization of SHE cells. Some of these published data are such that the frequency of spontaneous immortalization can be calculated (Barrett and Ts'o, 1978; Tsutsuyi and Ts'o, 1986; Newbold et al, 1982). The weighted mean of these calculations is $6.1X10^{-10}$. This indicates that the rate of spontaneous immortalization is indeed extremely low in SHE cells. This rate appears too low for a spontaneous mutation frequency. Therefore the question arises whether our data on induced immortalization should be explained by induced mutations. An alternative would be that immortalization, like some cases of malignant transformation, is the result of a two-step probabilistic process. Experiments directed at this problem are described in the next section.

2. Two-step probabilistic process in immortalization.

Methodology.

In a first experiment SHE cells have been treated with B(α)P (20 μg/ml) and 96 cultures have been initiated, each with 2000 cells. These cultures have been expanded and grown until senescence or immortalization occurred.

In a second experiment treated cells have been seeded for cloning and individual clones have been isolated and grown until the end of their lifespan, 62 control clones, 48 clones with a normal morphology from treated cells and 48 clones with a transformed morphology also from treated cells.

Results.

If the mutagenic treatment would lead to the direct induction of immortal variants in the population it can be calculated that only 0.4 of the 96 cultures would become immortalized. If, on the other hand, immortalization is due to a two-step probabilistic process in which treated cells have a fixed chance to become immortalized about 7.2 immortalized cultures can be expected. This expectation is based on a chance for immortalization of $5.2X10^{-8}$ per cell per generation, based on our previous experiments.

The number of observed immortalized cultures was 9 while 87 cultures were

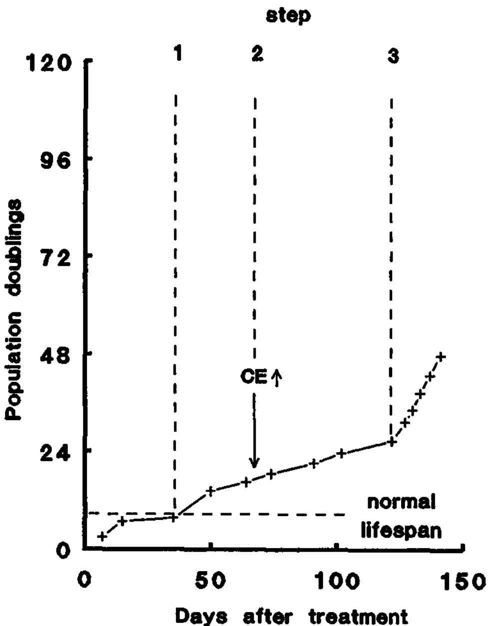


Figure 1. Kinetics of line BP-11 demonstrating three separate steps in immortalization: escape from senescence, increase in cloning efficiency and increase in growth rate.

exp. no	treat ment	no of cultures	step 1	step 2	step 3
1	B(α)P	18	2.2X10^{-4}	7.9X10^{-8}	2.5X10^{-8}
2	B(α)P	19	1.0X10^{-4}	1.1X10^{-8}	3.6X10^{-8}
3	X-ray	38	1.3X10^{-5}	6.6X10^{-8}	-------
4	X-ray	40	2.0X10^{-5}	2.3X10^{-8}	13.5X10^{-8}
5	ENU	18	1.1X10^{-4}	1.0X10^{-8}	2.6X10^{-8}
controls		64	<2.4X10^{-9}		
			weighted mean:	6.3X10^{-8}	4.3X10^{-8}

Table 1. Frequencies of steps in immortalization. Frequencies of step 1 are per viable cell; frequencies of steps 2, step 3 and controls are per cell per generation.

mortal. In the control cultures no immortalization was observed.
In the second experiment also no immortalization was observed in the cell populations derived from control cells while 3 populations derived from treated cells became immortal. All three were derived form clones with a transformed morphology. The expected frequency of immortal cultures was 0.002 for the hypothesis for direct induction and 4.2 for the hypothesis of induction via a two-step probabilistic process.

Discussion.
The results show that carcinogenic treatment does not lead to the direct induction of immortal variants but that the immortal variants arise via a two-step probabilistic process. Therefore it is indicated that the carcinogenic treatment leads to a kind of activation in the cells which is transmitted to the successive generations and which generates a chance of about $5.2X10^{-8}$ per cell per generation for immortalization. Similar phenomena have been described for the malignant transformation of C3H10T1/2 cells and appear present in the malignant conversion in the progeny of treated clonogenic cells from rat thyroid and mamma.
The implications of this finding are discussed in the "general discussion".

3. Characterization of newly immortalized cell lines.

Results and discussion.
a) Complementation analysis.
A TOR-mutant (resistant to thioguanine and ouabain) of an immortal cell line was fused with itself, with wild type cells or with eight different immortal cell lines. From each cross 25-40 proliferating hybrids were isolated and tested for lifespan. The fusion of the immortal line with itself led for 90% to immortal hybrids; fusion of the immortal line with mortal cells produced 75% mortal hybrids and fusion of the immortal line with the eight immortal lines produced a range from 25% to 100% of immortal hybrids.
Although in this way some evidence was obtained for complementation by wild type cells and lack of complementation by fusion with itself and for heterogeneity within the set of eight cell lines the results are not satisfactory because clearcut data on complementation groups are not obtained. The reason for this problem is sought in the long procedure for obtaining TOR-mutants which may alter the characteristics of the cell line. To solve this problem, experiments are being carried out in which the genes for neomycin-resistance and hygromycine-resistance are introduced into the cells by retroviral vectors and by using subsequently these dominant markers for the isolation of hybrids.
b) Chromosome analysis.
From nineteen immortalized cell lines the karyotype has been analyzed. Abnormal chromosome numbers were observed in all cell lines. Chromosome breaks were not observed except in one cell line in which double minutes were found in only two of the cells. In ten of the cell lines substantial numbers of diploid cells were still observed. Some cell lines had only chromosome numbers in the diploid range. There were no significant differences between cell lines which had only undergone two steps in immortalization when compared with the cell lines which had undergone the three steps.
From these observations it is indicated that aneuploidization is not the causal step in immortalization, nor tetraploidization but that

immortalization leads to genetic instability in the form of aneuploidization in the absence of chromosome breakage.

c) Growth in soft agar.

Thirteen lines have been tested for growth in agar by seeding the cells in a top layer of 0.33% agar over a bottom layer of 0.6% agar. Most lines did not grow at all and a few gave rise to only a few small colonies, the largest measuring 0.3mm in diameter. After cultivation of three lines over 16 more passages clonal growth in agar was observed.

From this it is concluded that newly immortalized cell lines do not grow in agar, which confirms published data.

4. Effect of tumor promotor on immortalization.

Methodology.

Parallel cultures of SHE cells have been started. Repeatedly half of the cultures received TPA (20 ng/ml) two days before they reached confluence and had to be passaged. The cultures were kept until senescence or immortalization occurred.

Results and discussion.

Four experiments have been performed with heterogeneous results: in two experiments no effect of TPA was observed and most of the cultures demonstrated senescence together with the control cells; the two other experiments demonstrated an extensive prolongation of the lifespan of the TPA treated cultures. The prolongation of the lifespan was about 2-4 fold for the TPA treated cultures.

The heterogeneity between experiments is not understood; it could not be correlated with differences in cell batch, serum source, population size or passage number. Still the results show that there must be conditions under which a tumor promotor can extensively enhance the lifespan of normal cells. As no immortalization was observed it could be calculated that in the presence of TPA the spontaneous rate of immortalization must be below 5.4×10^{-10} per cell per generation. As the spontaneous immortalization frequency is about 6.1×10^{-10} (see section 1) TPA does not enhance the spontaneous frequency of immortalization.

5. Effect of tumor promotor on genetic stability.

Methodology.

A population of mouse lymphoma cells with a low number of pre-existing mutants is generated by seeding 100 cells and growing them to about 4×10^{6} cells. Half of the cells are treated with the tumor promotor TPA (20 ng/ml) and subsequently a fluctuation analysis is started with both control and TPA-treated cells by seeding the cells in a large number of subpopulations of 100 cells each. From the fluctuation tests mutation frequencies per cell per generation are calculated which serve as indicators for genetic stability.

RNA was isolated from the TPA-treated cells and northern blots were prepared using probes to study the induction of fos A, fos B and collagenase.

Results and discussion.

Nine experiments have been performed. The pooled experiments lead to a mutation rate of 2.1×10^{-7} in control cells and 3.6×10^{-7} in TPA-treated

cells. Further analysis of the data indicates that the mutation rate could be up to 4-fold enhanced shortly after treatment, after which the mutation rate decreases to subnormal levels. These differences are however not significant ($0.05<P<0.10$). Experiments will be continued.
In the same cells TPA enhanced the expression of fos B and of collagenase 3-fold but no enhancement of expression of fos A was found.
These results show that TPA induces some of the genes of the signal transducing pathway in mouse lymphoma cells; genetic instability, if present, is mild and of short duration.

6. Expression of proto-oncogenes in successive stages of transformation of SHE cells.

Methodology.
Normal, immortal (BP-A) , ENU-transformed BP-A cells and BP-A cells transformed by the pEJ-cHa-ras oncogen were compared in growth in agar, in response to growth factors (PDGF and EGF), in tumorigenicity in nude mice and in the expression of five cellular proto-oncogenes (myc, p53, c-H-ras, erb-B and c-sis) to investigate whether the sequential steps in malignant transformation are characterized by a pattern of oncogene activation.

Results.
A high cloning efficiency in agar was observed for the transformed cells while the normal and immortal cells early after isolation did not grow in agar. Immortal BP-A cells gave rise to some colonies in agar after prolonged cultivation. Growth in agar correlated with tumorigenicity in nude mice: transformed cells gave rise to tumors, early immortal cells did not, while late immortal cells gave rise to tumors only after a long latency period. Further it was found that normal and immortal cells responded to the addition of external growth factors while an ENU-transformed line did not.
The level of c-myc transcripts was similar in normal, senescent, immortal or transformed cells and did not decrease in cells incubated in serum-free medium. The expression of p53 did increase in transformed cells, but this expression correlated with the expression of histon genes. The level of c-Ha-ras transcripts was similar in all stages of neoplastic conversion, except in the pEJ-induced transformant in which it was significantly enhanced. No erbB transcripts were detected and expression of c-sis was not observed in normal, immortal and pEJ-induced transformants. In 1 out of the 9 ENU-induced transformants expression of c-sis was detected.
Finally, southern analysis revealed no rearrangements in the five genes studied.

Discussion
C-myc and p53 were examined as it has been shown that they can play a role in immortalization; c-Ha-ras is known to be involved in transformation and c-sis and c-erbB were chosen because they code for a growth factor and growth factor receptor respectively and thus could be involved in the observed loss of growth factor requirement of the transformed cells.
The results indicate that c-myc is not involved in the immortalization or transformation of SHE cells. As the increase in p53 transcription correlates with transcription of histon genes it is most likely that this increase is largely due to the increase in number of cycling cells. It is also concluded that c-Ha-ras, c-erbB and c-sis do not play a predominant role in immortalization or transformation of SHE cells.

General discussion.

The results obtained in this project taken together with some scattered data from the literature lead to some rather important considerations on tumorigenesis:

Origin of tumor cells.
Malignant transformation is known to be a multiple step process. The number of steps involved, as indicated by population studies on the age related incidence of tumors, is thought to be 4-6 for most types of tumors. The nature of the steps is largely unknown nor the frequency with which they occur but there are strong indications that genetic events are involved. This follows from the changes found in familial cancer in which two well known types of genetic events appear causally related to the origin of the cancers, one type leads to the inactivation of a gene and a second leads to the loss of heterozygosity for the affected allele. As the same types of genetic alterations are observed in similar non-familial cancers similar types of origin are ascribed to those tumors. Moreover the presence of activated oncogenes in most tumors, which have arisen by a wide scala of genetic alterations also suggest genetic alterations as a cause for the origin of tumors. This notion is further supported by the fact that DNA-damage is the principal cause in the origin of tumors. Therefore the predominant model for the origin of tumors is that the mutations of genes are the causal events and that the multiple steps consist out of accumulated genetic alterations. This model on the origin of tumor cells can be adequately described as the stochastic genetic model.
The findings of this project are not consistent with this model. Malignant conversion of primary SHE cells is also here a multistep process. We found that immortalization itself requires at least three steps, data in the literature indicate that conversion to growth in agar is a fourth step and there are some indications that a fifth alteration is needed to evoke tumorigenicity in cells which are able to grow in agar. The necessity of 4-5 alterations in order to obtain tumorigenicity is in agreement with the stochastic genetic model. However the findings do not fit this model in two ways:
Firstly all evidence suggests that the observed steps are sequential and that each step is a prerequisite for the next: escape from senescence is required for the increase in CE, increase in CE precedes increase in growth rate; immortality (the result of the three foregoing steps) is a prerequisite for growth in agar. A strict sequence of events has sofar not been observed in the gene alterations of tumorcells and there are several indications that activation of a certain oncogene or inactivation of a suppressor gene can be as well an early as a late step in the history of a tumor.
Secondly there is evidence that the different steps are not directly induced by the carcinogen but that they arise by an indirect mechanism. This is shown in this project for the very first step, it is indicated by the data of Crawford et al for the fourth step, it is shown by Kennedy et al for the change of immortal C3H10T1/2 cells into malignant cells and it is indicated for the malignant conversion of progeny from thyroid and mamma clonogenic cells in rats.
Therefore it cannot be excluded that all the sequential steps are indirect and that often, instead of mutations leading to carcinogenesis, a

carcinogenic process is initiated which leads to the stepwise events. The events although not mutations still might be genetic e.g. gene amplification or somatic recombination. Our results and many literature data on euploidy of immortal cells seem to rule out the possibility that one of the steps is aneuploidization.
It is conceivable that the observed events in the multistep process of malignant conversion of primary SHE cells are epigenetic in nature. A model on the origin of cancer cells based on epigenetic changes could be adequately described as the sequential epigenetic model.
A sequential epigenetic model of malignant conversion is still compatible with the stochastic genetic model: tumorigenicity can be considered as a perturbation of the signal transduction systems in the cell and both genetic and epigenetic changes will be able to affect these systems. As DNA damage is known to induce a cascade of gene activations and gene inactivations the whole signal transduction system can be involved. From studies with x-irradiated C3H10T1/2 cells it is known that some changes persist over more than 25 generations after treatment; thus it is conceivable that stable epigenetic changes might occur with a neoplastic implication.
As postulated by Herrlich it is also possible that the switching on of a signal transduction pathway leads to genetic instability and that in turn to the (in)activation of oncogenes. In this view the stochastic genetic model still predominates. Sofar our studies on the induction of genetic instability by the tumor promotor TPA indicated that the switching on of a signal transducing pathway leads to only a mild genetic instability if at all; this point of course needs further exploration.

Cellular lifespan as rate limiting factor in carcinogenesis.
Until now there is no evidence for gross species specific differences between cell lines from different mammalian species in sensitivity to mutagens and in induction of genetic alterations. If tumors arise solely by the accumulation of genetic alterations there would be for somatic cells from different species no gross species specific differences in frequencies of malignant transformation. However such differences are likely to exist between mouse and man: based on the difference in cellular mass (25g versus 60000g) and the difference in lifespan (2.5y versus 75y) between mice and men it can be estimated that the rodent somatic cells have a 70,000 higher chance to give rise to a tumor cell than human somatic cells. This figure is based on the supposition that a mouse population generates the same amount of tumors per individual as a human population. Although such a supposition is not necessarily exactly true the argument of the species specificity probably cannot be invalidated.
Sofar the stochastic genetic model has no implicit explanation for species-specific frequencies in tumor cell formation. On the other hand epigenetic phenomena have almost by definition species specificity (e.g. differentiation).
As carcinogenesis is a multistep process, for each step a rare progenitor cell has to grow to a large population in order to generate again a rare variant cell for the next step. As the frequencies of four successive steps in malignant conversion of primary SHE cells are known from our experiments and from data of Crawford et al it can be calculated how many cellgenerations are minimally necessary to generate a tumor cell. This calculation leads to about 97 population doublings. This figure exceeds by far the normal lifespan of normal cells, both from rodent and human origin. From this it can be concluded that increase in lifespan of cells is a prerequisite in the process of formation of a tumorcell. We found

that treatment with carcinogens in general reduced the lifespan of cells and that probably all the first three steps of the multistep process increased the lifespan. Moreover it was observed that there are conditions in which the lifespan of SHE cells can be strongly increased by the tumor promotor TPA.

If immortalization is a prerequisite for tumor formation and if epigenetic mechanisms play an important role in immortalization it is understandable that wide species-specific differences exist in tumor cell formation: normal human fibroblasts and epithelial cells do not immortalize spontaneously and are very hard to immortalize by carcinogens; only a few cases have been described. Rodent cells on the other hand are easy to immortalize and mouse fibroblasts even immortalize spontaneously at a high frequency. If immortalization of mouse cells would be also a multistep process the frequencies of the steps have to be orders of magnitude lower than mutation frequencies. Therefore the immortalization frequencies correlate with species specific differences in malignant transformation and therefore immortalization should be considered as the rate limiting step in carcinogenesis. Apparently there is a gap in our knowledge at this point.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

Department of Molecular carcinogenesis, University of Leiden. (Prof. Dr. A.J. van der Eb).

V. Publications:

Kok, A.J. de, H.M. Sips, L. den Engelse and J.W.I.M. Simons. Epidermal growth factor enhances N-ethyl-N-nitrosourea induced morphological transformation of Syrian hamster embryo cells. Carcinogenesis 9 661-664 1988

Kok, A.J. de, H. Sips, L. den Engelse and J.W.I.M. Simons. Prolonged in vitro exposure of Syrian hamster embryo cells to 3-aminobenzamide induces transformation and chromosomal alterations but not gene mutations. Carcinogenesis 10 237-240 1989.

Bols, B.L.M.C., J.M. Naaktgeboren and J.W.I.M. Simons. Sequential steps are required for full immortalization of Syrian hamster embryo cells. "In Cell Transformation and Radiation-induced Cancer" , Edts. K.H. Chadwick, C. Seymour and B. Barnhart. A. Hilger, Bristol and New York 1989 109-116.

Simons, J.W.I.M., B.L.M.C. Bols and J.M. Naaktgeboren. Immortalization as asn an endpoint in studies on malignant transformation. Proc. of the Fifth Intern. Conf. on Environ. Mutagens. Alan R. Liss, Inc. New york, N.Y. 1990 (in press).

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-093-IRL

Federated Dublin
Voluntary Hospitals
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Head(s) of research team(s) [name(s) and address(es)]:

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Title of the research contract:

Radiation response of the thyroid : survival and alteration towards malignancy in cell culture and human systems.

List of projects:

1. Studies of radiation induced effects on thyroid cell survival and function, and of the dosimetry of radioiodine in cell cultures.
2. Carcinogenic aspects of thyroid irradiation.

Title of the project no.: 1

Studies of Radiation Induced Effects on Thyroid Cell Survival and Function, and of the dosimetry of radioiodine in cell cultures.

Note: A combined report on the achievements in Projects 1 and 2 (over the last four years) is presented below, as the material in both is intimately related.

Head(s) of project: J. F. Malone.

Scientific staff: A. Murphy, J. F. Malone, C. Mothersill, N. Sheahan, K. Maher, M. Lewis, B . Tuohy.

I. Objectives of the project:

When established in 1985 this project had two objectives. First to study the biological responses (other than neoplastic development) to irradiation, and secondly to attend to the necessary developments in dosimetry. The first study concentrated on the survival, recovery and function of thyroid cell cultures after irradiation. These studies contributed to the understanding of non-malignant radiation induced thyroid diseases, as well as providing correlative information for the carcinogenic studies in Project 2, as both sets of gross endpoints are strongly interrelated. They also indicate if the status of the thyroid in vivo might be manipulated to prevent undesirable radiation sequelae. The dosimetric studies involve detailed micro dosimetric monitoring of the structure of thyroid cell cultures using a sophisticated computer model and image analysis computer, as well as microscopic and macroscopic experimental investigations to confirm these results.
During the Project additional investigations in dosimetry, in modelling the thyroid radiation response, in the response of human cells in culture, and on the r.b.e. of 131-I were undertaken.

II. Objectives for the reporting period:

(a) Study of the survival and recovery of sheep thyroid cells after gamma rays administered in single or multiple doses with a view to preparing the way for 131-I irradiation studies.

(b) Study and interpretation of the response of sheep thyroid to 131-I.

(c) Modelling response of thyroid to irradiation in clinical, in vivo and cell culture systems.

(d) Study of radiation response of human thyroid cells in culture.

(e) Dosimetric and microdosimetry studies of 131-I in various sizes of thyroid from experimental animals and the foetus to the adult human gland.

(f) Microdosimetric calculations for cell cultures.

(g) Methodological developments for cell cultures.

Title of the project no.: 2

Carcinogenic Aspects of Thyroid Irradiation.

Note: A combined report on the achievements in Projects 1 and 2 (over the last four years) is presented below, as the material in both is intimately related.

Head(s) of project: J. F. Malone.

Scientific staff: A. Murphy, J.F. Malone, C. Mothersill, N. Sheahan, M.K. O'Connor, M. Lewis, B. Tuohy, P. Gilligan.

I. Objectives of the project:

When established in 1985 it was hoped to determine and quantify the carcinogenic effect of acute and radioiodine irradiation to the thyroid using differentiated cell culture transformation models, and if possible human material. It was also hoped to quantify the dose response relationship with respect to the induction of specific features of transformation in thyroid cells and to determine if 131-I iodine taken up by follicles contributed to transformation.

In addition it was hoped to explore the low dose and low dose rate effects and where possible, determine a reliable 'r.b.e.' value for 131-I. Finally it was intended to continue to support epidemiological studies of radiation carcinogenesis and related initiatives, being co-ordinated through this programme.

II. Objectives for the reporting period:

(a) To identify indices associated with cell transformation and develop and apply them to thyroid cell cultures.

(b) To quantify the dose response for indice(s) of transformation after high dose rate gamma and low dose rate 131-I irradiation.

(c) To identify and highlight important changes relevant to dosimetry calculations.

(d) To calculate the dose to foetal neurological tissue from 131-I in the foetal thyroid.

(e) To develop methodology for assay of human cells and of small volumes of tissue in culture.

(f) To arrive at a figure for the r.b.e. of 131-I and identify the mechanisms underlying the figure identified.

(g) To continue collaboration in epidemological studies.

III. Progress achieved:

As already mentioned the final reports for Project 1 and Project 2 have been grouped together as the material in them is closely interrelated. This permits the presentation of a more coherent report in which each topic is fully developed once, rather than being divided somewhat arbitrarily and being presented twice, which would otherwise be necessary. The headings under which the report is presented are:

1. Introduction.
2. Methodology.
3. Results/Discussion:

    3.1. Survival and Recovery Studies in Sheep Cells.
    3.2. Survival and Recovery Studies in Human Cells.
    3.3. The r.b.e. for 131-I in Survival Investigations.
    3.4. General Developments in Methodology.
    3.5. Unified Model of Proliferative Aspects of Thyroid Radiation Response.
    3.6. General Developments in Dosimetry.
    3.7. Foetal - Neurological Dosimetry.
    3.8. Absorbed Dose as an Index of Harm.
    3.9. Microdosimetric Methodology.
    3.10. Assays for Indices of Cell Transformation.
    3.11. Dose Response Relationship for Indices of Cell Transformation.
    3.12. 131-I and Human Investigations.

4. Conclusions.
5. References.

1. INTRODUCTION.

Interest in the radiation response of the thyroid arises from the fact that it is likely to be exposed in many situations. These frequently involve its avidity for radionuclides of iodine, such as 131-I. This is particularly important in the aftermath of nuclear accidents which release abundant quantities of radioiodine, in medical diagnostic and therapeutic applications, and in health physics aspects of survillance of many medical and laboratory workers. The consequences of thyroid irradiation include induction of benign and malignant neoplasia which may be fatal but more frequently are not. In addition hypothyroidism may be induced, which is consequential on disruption of cell division. Much of the study of thyroid radiobiology has been restricted by the absence of good experimental models, a suitable theoretical framework, and inadequate dosimetry. This report presents detailed studies of cell survival which are dosimetrically sound and within the mainstream of radiobiological studies of other tissues. These studies are linked in a well developed conceptual approach that integrates them with previous apparently anomolous results and well established clinical data. In addition reports on new developments on the biological effectiveness of 131-I, dosimetry, including some special foetal concerns, and studies of cell transformation in cell cultures.

2. METHODOLOGY:

2.1. In general the methods used for the investigations described here have been set out in detail elsewhere and are only referred to here in summary form. Where necessary for clarity reference to a particular feature of the method used is made in the Results/Discussion Section.

2.2. Cell Cultures:

The methods used have been described in detail in O'Connor et al (1980), and in some of the publications listed for this report (Seymour et al., 1987). The methods for human cells were simple adaptations of those developed for the sheep system. Experiments were generally initiated on well differentiated confluent cell cultures 10 to 15 days after explantation.

2.3. Radiation and Subculture:

External radiation was generally administered using ^{60}Co gamma radiation at a dose rate of the order of 1 to 2 Gy min^{-1}. This necessarily varied over the duration of the workprogramme. Irradiation with 131-I generally took place on cultures of similar age to those used in 60-Co experiments. Appropriate amounts of 131-I were added in the form of Na.I to the culture medium and left in place for a period of one week. After the final irradiation or removal of 131-I, the confluent cells were subcultured and assayed for cell survival, index of transformation, or other endpoint as appropriate.

2.4. Survival Assay:

Cell survival was assayed using the clonogenic method first described by Puck and Marcus (1956) and adapted for use with thyroid cultures by O'Connor et al (1980a, b). Briefly flasks were plated with known numbers of cells after irradiation. They were incubated for 10-12 days which allowed development of macroscopic colonies from single cells. The number of macroscopic colonies growing were scored and compared with controls. It was particular important to control for the variability in plating efficiency with innoculated cell number.

2.5. Indices of Transformation:

These required serial subcultures of cells which were achieved by detaching the cells and replating them at a concentration of about 1×10^6 cells in 5ml. of growth medium. The cultures were then grown to confluence or until growth ceased. Senescence was judged to have taken place in some investigations when subcultures stopped growing and in others by assessing the plating efficiency of the cells at each subculture. The latter approach had the advantage of allowing quantitative determination of the number (if any) of cells giving rise to immortal clones and of allowing microscopic exclusion of material fibroblast in origin.

Loss of contact inhibited growth was determined by counting any foci developing on the confluent monolayer prior to subculture. After senescence of controls the cells for assay (irradiated or otherwise) were tested for soft agar growth using a method based on that of Kruse and Patterson (1973). In some experiments foci and or soft agar clones were subcultured to check for characteristic thyroid function and/or epithelial morphology. Thyroid function in primary cultures and subcultures was determined using the methodology described in detail in O'Connor et al., (1980a). Ability to trap iodine, and T_4 production, were accepted as

criteria of thyroid origin.

LDH isozyme concentrations of the five isozymes of lactate dehydrogenase were determined using gel electrophoresis according to the method of Market and Moller (1959).

2.6. Dosimetry:

Dosimetry of external 60-Cobalt irradiation used the standard techniques applied for radiotherapy. Dosimetry of 131-I in cultures was based on the assumption of uniform distribution of the 131-I in the medium. The cells were assumed to be irradiated on the flat plane through the centre of an infinite hemisphere of material throughout which the concentration of 131-I was equal to that in the medium.

Microdosimetric modelling of the thyroid was performed using methods previously described. To facilitate calculations, should they become necessary for short range radiations a method using a nuclear medicine image analysis system was adapted for acquisition of data on follicle dimensions.

Calculation of doses to the neurological system of the foetus from 131-I in the foetal thyroid was performed using standard physical data and foetal anatomy derived from England, 1983.

General aspects of dosimetry were approached from standard accepted physical and biokinetic methods (Malone, 1980).

3. RESULTS AND DISCUSSION.

3.1. Survival and Recovery Studies in Sheep Cells:

3.1.1. O'Connor et al. (1980) published an extended account of the properties of differentiated sheep thyroid cells in culture and a methodology that could be employed to use the cultures for radiobiological investigations. In contrast to the in-vivo systems previously studied it was possible to obtain sufficient proliferation to develop a clonogenic assay, although the plating efficiency was relatively low. Growth curves demonstrating 10 cell divisions were reproducibly obtained and plating efficiencies were generally in the range 0.1 - 0.4 per cent, though higher values occured.

3.1.2. The first reported survival curve for this system (O'Connor et al., 1980) gave a Do value of 4.1 Gy, and thereby appeared to support the large value seen in the gland weight assay (see below). In retrospect this value has been demonstrated to be untrue, due in part to the fact that survival was followed for one decade only. Obtaining a reliable value was difficult due to the low plating efficiencies and other technical problems, particularly those associated with disaggregation of clumps of cells. Resolution of these problems led to the establishment of an unambiguous value of the Do for sheep thyroid cells in the range 1.5 - 2.0 Gy (Fig. 1) and an extrapolation number in the range 5-20. Split dose experiments in which the two doses were separated by 0-6 hours demonstrated significant recovery with a plateau value approaching 5. These values are supported by data from the transplantation assy for thyroid cells of De Mott et al., (1979).

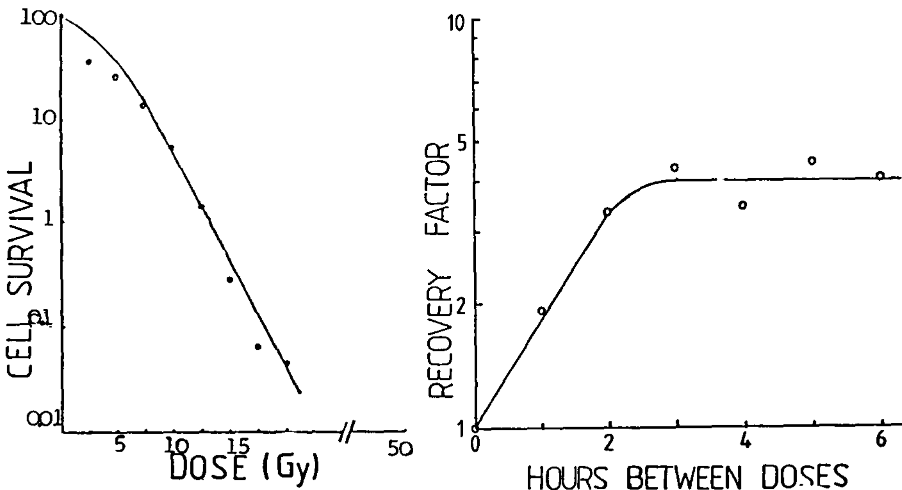


Fig. 1(a) illustrates typical Co^{60} survival curve using the clonogenic survival assay.

(b) curve illustrating recovery between doses of radiation.

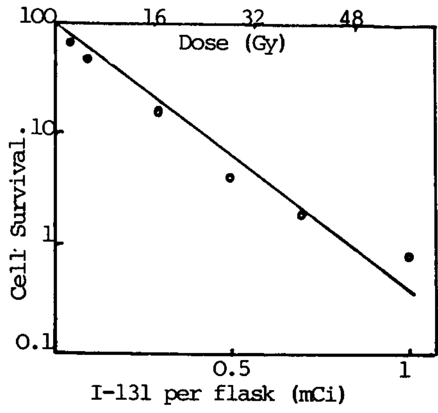
3.1.3. Considering colonies of fibroblast origin in the total surviving fraction of single dose survival curves, after 60-Co irradiation, had little or no effect on the Do values obtained, but did result in a notable alteration of shoulder size, with extrapolation numbers range narrowing to 17-21. However during split dose experiments no significant effect on either Do values or extrapolation numbers was observed on inclusion/ exclusion of fibroblast colonies in the total survival level. Table 1 shows the mean level of fibroblast colonies obtained during single dose survival experiments, for 60-Co irradiated sheep thyroid cells, expressed as a percentage of the total colonies counted.

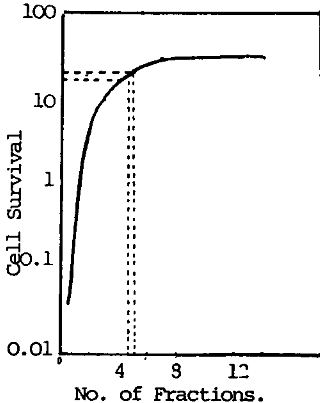
Table 1: Percentage of fibroblast colonies obtained on irradiation of sheep thyroid cells with acute doses of 60-Co.

Dose (Gy)	0	1.5	2.5	5	7.5	10	12.5	15
Fibroblast Colonies (%)	11.45	12.9	10.9	13.9	14.7	19.3	19.1	19.9

3.1.4. In an experiment involving multifractionation of a 14Gy dose of 60-Co the expected increase in survival with increasing number of fractions was observed, with little increased recovery between 7 and 14 fractions per week. (See Fig, 2).

FIGURE 2.





(a) Average of cell survival for 5 sheep thyroids after I-131 irradiation for ~1 week, and

(b) Surviving fraction of sheep thyroid cells versus number of fractions of a 14 Gy dose of 60-Co.

3.1.5. Similar methods were employed to produce survival curves for sheep thyroid cells after 131-I irradiation distributed over one week. The Do obtained was of 7-10 Gy. (Fig. 2). In these studies the uncertainties inherent in 131-I dosimetry in vivo were eliminated and hence the dose comparisions may be taken as reliable. A comparison of this data with the

multifraction experiment shows that with larger numbers of fractions the survival values were greater than those for 131-I. These results agree with previous comparisons and indicate that dose rate alone could account for the 131-I effect.

3.2. Survival and Recovery Studies in Human Cells.

3.2.1. Survival curves for thyroid cells of human origin were much more varied than those from sheep. For 60-Co gamma rays the Do was in the range 0.5 - 2 Gy and the extrapolation number was close to unity. In Fig. 3 survival curves from four human thyroids are presented. While all four have a different survival pattern they are similar in that each is much more radiosensitive than sheep thyroid. This confirms a prior, but unexplained, observation of Clifton et al.(1978). In Fig. 3 two survival curves for human cells after 131-I irradiation are presented. The Do values are 6 and 9 Gy.

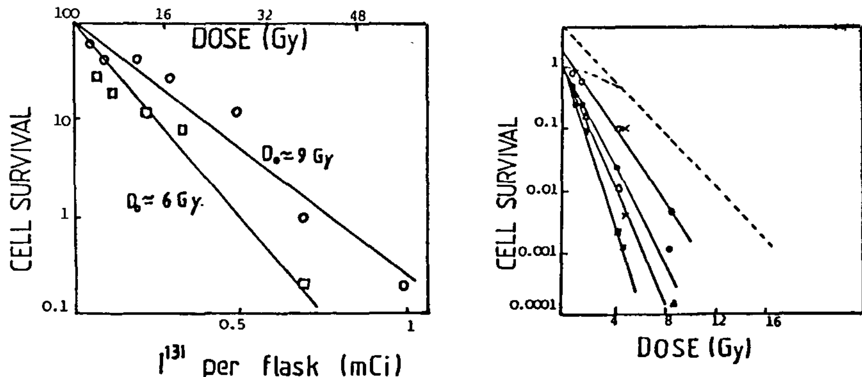


Fig. 3 (a) Cell Survival for two different Human Thyroid Cultures after 1 week of 131-I irradiation, and
(b) Survival curve for Human Thyroid Cells from 4 different patients. The dotted line is the survival curve for sheep cells.

3.3. The r.b.e. for 131-I in Survival Investigations.

3.3.1. From the above it is clear that the r.b.e. of 131-I as assessed in the sheep thyroid assay is 0.2 when the ratio of Do values is taken, i.e. 10Gy for 131-I compared to 2 Gy for high dose rate gamma rays. The value for human cells similarly assessed is 0.16 - 0.20 which, for practical purposes is the same. It is of interest that the 131-I/60-Co ratio remains constant despite the significant increase in radiosensitivity of human cells. The above values compare with a value of 0.1 determined using the goitrogen induced gland weight increase assay for cell survival in rat thyroids (see 3.5. below). However this value is subject to substantial dosimetry errors due to the methods used to assess the dose to the thyroids. Values ranging from 0 to 1 are cited for the r.b.e. for 131-I for cell transformation/carcinogenesis in Section 3.12 below.

3.4. General Developments in Methodology.

3.4.1. A study was undertaken to determine if small numbers of thyroid cells can be cultured. The results were interesting and indicated that when "well culture" methods were applied, the relatively small numbers of

cells from a needle biopsy can be cultured successfully. However the yield, in terms of cell number, from such cultures is small, when the needs of radiobiological experiments are taken into account.

3.4.2. Because of the above an alternative approach to culture of small amounts of tissue was also developed. This involved growth of intact explants and monitoring the outgrowth of cells from them. This technique has been successfully employed by other groups. The explant technique gives rise to growth inhibition curves for thyroids which are highly resistant and have Do values similar to those previously found by Malone et al. using the gland weight assay (see Section 3.5. below). The difference between these results and the results of the clonogenic assay suggests that only a small fraction of cells which grow out from the explant are involved in significant proliferation.

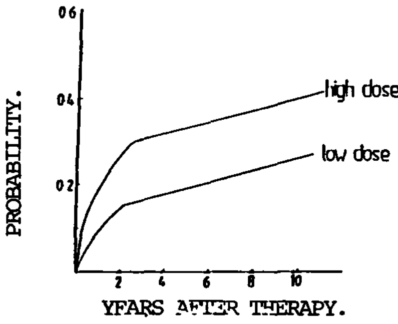
3.5. Unified Model of Proliferative Aspects of Thyroid Radiation Response.

3.5.1. Numerous studies have been performed on different aspects of the radiation response of the thyroid (Malone, 1975; Dumont and Malone 1980). These results have used different methods of assessing thyroid cell function and response, e.g. clinical hypothyroidism (Malone and Cullen, 1976, 1977); transplantation assay (Clifton et al. 1978; De Mott et al, 1979); weight assay (Greig et al, 1969, 1970; Malone 1975); and clonogenic survival assays (O'Connor et al, 1980 a,b,c). Using this literature it is extremely difficult to correlate the information presented, into a single coherent self consistent picture. In fact it appears from that the various responses of the thyroid contradict each other. These apparent contradications need to be addressed to allow a model for the thyroid at the cellular and macroscopic levels be developed and correlated.

3.5.2. A model has been developed for the cell population structure of the thyroid in line with those used for other tissues, such as the haemapoietic system, in which cell kinetics and radiation response are correlated. The model is based on the "H-F Hybrid" type of Wheldon and Miehalowski (Wheldon, et al 1982). It proposes three population types in the thyroid, each of which can be correlated to a significant degree with a well defined and assessable feature of the radiation response. To present the model coherently requires reviews of the background information with respect to (a) the induction of Hypothyroidism after radioiodine therapy; (b) the assay of cell survival using the gland weight increase assay; (c) the clonogenic cell survival data presented above; and (d) the available cell kinetics data. This is undertaken in the following paragraphs.

3.5.3. Hypothyroidism after Radioiodine Therapy:

Shortly after the introduction of 131-I therapy for hyperthyroidism it became evident that some of those treated would become hypothyroid. Thereafter it was demonstrated that the fraction of those treated who subcumb to hypothyroidism increases with time (see Fig. 4).



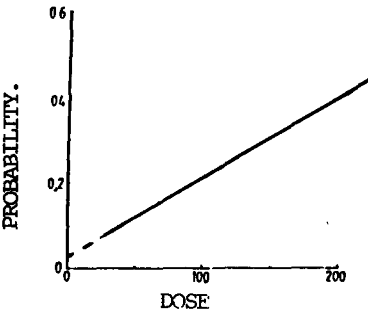


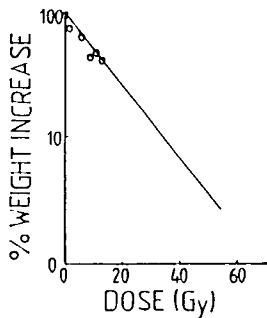
Fig. 4: Curves for the probability of hypothyroidism (a) after I-131 therapy as a function of time, and (b) as a function of dose 2 years after therapy.

For many years it was felt that incidence bore little relationship with radiation dose. Malone and Cullen (1976) demonstrated a direct linear dose-effect relationship for the early component of hypothyroidism, i.e. that prior to the shoulder on the curve in Fig. 4 (a). This relationship is demonstrated in Figure 4 (b) and is quite convincing. It was subsequently confirmed by (Maxon et al.,1977) for 131-I and by Malone et al. (1977) for 125-I.

3.5.4. The time scales involved are consistent with damage to the mechanisms of cellular proliferation (Table 2) which will eventually express themselves as a reduction in organ function, due to a depletion in population. This mechanism has been proposed, in conjunction with others over the years, but has not been fully accepted due (among other reasons) to the absence of any apparent relationship between the doses involved and those involved in cell survival or related studies. For example the dose required for induction of a certain fraction of early hypothyroidism is evident in Fig. 4. For an incidence of 0.5 (i.e. 50% of the exposed population) a dose of 250 Gy is required. This seems very large but is confirmed in the U.S. Health Effects Model Study for the Biological Consequences of a Nuclear Incident (Evans et al. 1985).

3.5.5. Cell Survival using the Gland Weight Increase Assay:

The thyroid was a late starter with respect to the measurement of cell survival and other aspects of radiobiological assessments. This was in part due to the relatively small amount of cell proliferation that takes place in the gland, and to the absence of suitable in-vitro models. Early attempts at studies which mimicked cell survival used a goitrogen stimulated weight increase in the gland (Malone 1975), almost all of which was due to cell proliferation and attributable to, at most, 2 - 5 cell division. The goitrogen stimulated weight increase is sensitive to radiation. It decreases with dose until a plateau value of approximately 10% is reached at a dose of the order of 20 Gy. This corresponds to the loss of all proliferative function in the cells, and the small component of weight increase seen was due to hyperthyrophy alone (Malone 1975, Dumont et al. 1980). Thus this would correspond with the tolerance limit for a single acute dose of radiation and is in keeping with the figures for thyroid cited in the radiotherapy literature (Rubin & Cassaret, 1968). This figure is therefore a key constant for the thyroid and hence must be included in any model and is listed in Table 3.



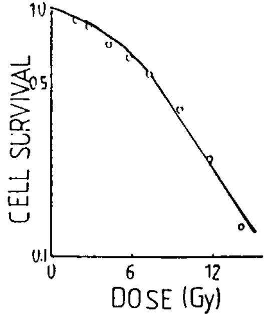


Fig. 5: (a) Weight increase using the fat pad assay.
(b) Inhibition of goitrogen - induced weight increase in the rat thyroid by a single dose of x-rays expressed as cell survival.

3.5.6. "Cell Survival" was calculated by subtracting the non-proliferative component of weight increase from the total and ploting the remainder (normalized to 1.0 or 100 per cent for controls). This gave a classic exponential survival curve with a shouldered region (Fig.5), which was further attested to by the existence of a split dose recovery response. The Do of this survival curve was generally the order of 4-5 Gy which was attributed to some or all of the follcwing: (a) the unusual radioresistance of the gland; (b) the fact that the assay was based on the first few post irradiation cell divisions which are normally more radioresistant than later divisions; (c) the fact that the assay was based on overall cell numbers rather than survival; (d) numerous other ingeneous explanations. In retrospect the explanations most likely to be true are (b) and (c) and there is now little reason to attribute unusual radioresistance to the gland. Hence (b) and (c) are here accepted as the main explanations for the 5 Gy Do value in Table 3 and Fig. 5(b).

3.5.7. Further experiments with the weight assay demonstrated considerable recovery in multifraction experiments (Malone et al. 1974). Extrapolating, these results to the point at which no residual proliferative capacity would be left in the gland, gives a value of about 50Gy for the point in question (Malone et al. 1974, Malone 1975). This corresponds to the endpoint, which in the case of a single dose of 20 Gy we equated with tissue tolerance. With regard to protracted distribution of dose over time "survival curves" using the weight assay also exist for 131-I in the rat thyroid with a Do of about 55 Gy (Dumont et al. 1980). However great uncertainty attaches to the 131-I dosimetry in these studies.

3.5.8. Cell Kinetics Data:

Cell Kinetics Data for the thyroid are not widely available. Nevertheless sufficient information is available from in vivo and in vitro sources to be able to directly state or infer many features of the cellular heirarchy and kinetics structure in the gland. The available data necessary for the considerations to be taken up here are summarised in Table 2 which have been drawn from various sources (Malone et al., 1983; Malone 1975; O'Connor et al. 1980; Greig et al., 1970; Redmond et al., 1979, 1980). Under normal conditions the rate of cell turnover is low. The mitotic and labelling indices are 3.5 and 7.8 cells per 10,000 respectively in rodents.

TABLE 2: Contains typical cell kinetics parameters, namely in rodents.

TABLE 3: Summary of the different response of the 3 levels of the thyroid model.

TABLE 2.

Parameter	Value	Range
Mitotic Index	3.5/10,000	0.07-11.9
DNA Labelling Index	78/10,000	15 - 180
S Phase Duration (Labelled M)	7.5 - 10 hours.	
S Phase Duration $(\frac{LI}{MI} \times 0.5)$	11 hours	
Cell Cycle Time (Normal)	> 9 - 16 weeks	
Cell Cycle Time (Stimulated Growth)	2 - 6.25 days	
Weight Doubling Time	3 - 7 days	
Growth Fraction Stimulated	0.3 - 1.0	
Plating Efficiency	0.1 - 5%	
Doubling Time in Culture (Stimulation)	1 - 2 days	

Cell Kinetics Parameters, mainly in Rodents.

TABLE 3.

Endpoint	Quantitative Feature Used	Dose (Gy)
Hypothyroidism	"Early Incidence in 50% of Population"	~ 250
Weight Assay	"Do"	~ 5
	$D_{(Tol.)}$ (Single Dose)	~ 20
	$D_{(Tol.)}$ (Fractionated)	~ 50
	$D_{(Tol.)}$ (Radioiodine)	~ 55
Clonogenic	"Do" (Sheep Cultures)	~ 2
	Do (Fat Pad)	~ 2
	Do (131-I Sheep Cultures)	~ 10
	Do (Human Cultures)	~ (0.8 - 1.5)
	Do (131-I Human Cultures)	~ 6 - 9
Hypothyroidism	Early Incidence in 50% of Population but Corrected for Biological Efectiveness of 131-I (i.e. 250Gy x 0.2)	~ 25-50

On this basis the lower limit for cell turnover time is the order of 9 weeks but it is probably longer, and 16 weeks or greater appears to be a reasonable estimate for small animals. For larger species with a longer lifespan it is correspondingly longer, and for example, Dumont estimates it is about 8-10 years in humans (Dumont, 1989). The duration of the S phase from various considerations including labelled mitosis curves is about 7-11 hours. The resting phase of the gland can be interupted by goitrogenic stimulation which results in growth of the gland. During growth the DNA labelling index increases to about 5 per cent and the cell cycle time can be inferred to lie somewhere between the extremes of 2 and 6.25 days. From these and related considerations it may be inferred that the growth fraction during goitrogenic stimulation lies between 25 and 100 per cent. The higher figure is unlikely because of the fact that all the cells eventually reach a stage where no further mitosis is possible, and hence it is quite likely that some of the cells are in this state even in the beginning of stimulation. As the overall population increase is a factor of about 10, the number of divisions per mitotically active cell must be between 3-4 (for 100% growth fraction) and 5-6 (for a 25% growth fraction) (Malone et al., 1983). This upper limit of 5 divisions appears to be the maximum available during the adult lifespan of any species so far assessed.

3.5.9. Thyroid cells maintained in primary culture do not normally exhibit much proliferative activity when they are kept in a monolayer or near monolayer form. However if the cultures are diluted to encourage essentially clonogenic growth a small fraction of the cells between 0.1 and 5 per cent will plate and grow to viable clones undertaking 10 or more cell divisions. This small fraction of cells with large, if not unlimited division potential, have a doubling time, the order of 1-2 days which also sets them apart from the normal proliferative/goitrogenically stimulated proliferative response of the bulk of the cells in the gland referred to above and set out in Table 1, (O'Connor et al., 1980).

3.5.10. Proposed Model: From the above data it is evident that the radiation response of the thyroid has been evaluated at three levels i.e. clinical hypothyroidism; survival as assessed by the weight assay; and clonogenic cell survival. It is also clear that the doses required to produce the various responses seem to be, superficially at least, at odds with each other. The cell kinetic data suggest that the thyroid population may be broadly classified as an H-F type (Wheldon et al., 1982). As illustrated in Fig. 6 it is reasonable to regard it as consisting of three components consisting of (a) precursor/stem-cell types; (b) mature/maturing cells with limited division capacity; and (c) mature metabolically functional cells whose division capacity has been exhausted. The proportion of the population in each of these categories has not been exactly determined, but from the information presented above limits on each compartment may be placed. Thus it is reasonable to postulate that the precursor/stem cell compartment will contain only the cells capable of proliferation to a level that renders them clogenic, i.e. 0.1 to 5 per cent. It is the behaviour of this compartment that is seen in clogenic cell survival curves.

3.5.11. The second compartment of mature/maturing cells with some division potential must consist of the growth fraction of the cells in the gland when it is goitrogenically stimulated (less the content of Compartment 1). This is clearly between 25 and 100 per cent (Table 2) less something between 0.1 and 5 per cent. Thus Compartment 2 consists of between 24.9 and 99.9 per cent of the cells, which have a limited division

capacity of something between zero and about 5 divisions per cell. It is the behaviour of this compartment that is seen in the weight assay. The apparent radioresistance in this assay is conferred by the fact that it is based on the early post irradiation cell divisions.

The third and final compartment consists of cells which are metabolically active, have no residual division capacity and hence will eventually die. Between 0 and 75 per cent of the cells must lie in this compartment and its behaviour determines the post irradiation appearance of hypothyroidism. Thus the model proposes that the thyroid consists of three population types each associated with a specific feature of the known radiation response.

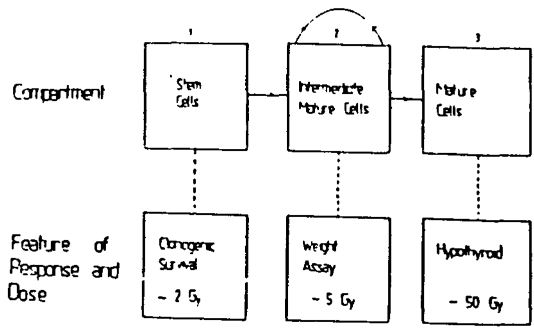


Fig. 6: Model for the response of the thyroid to irradiation.

3.6. GENERAL DEVELOPMENTS IN DOSIMETRY.

3.6.1. The ICRP values, and values calculated using the MIRD scheme of thyroid dosimetry, both of which were widely used to estimate radiological detriment post Chernobyl, are no longer adequate, and fall down in a significant number of areas. The physical and physiological assumptions used regarding the thyroids metabolism, shape, mass and iodine uptake distribution and kinetics are poorly correlated with those of a non homogenous population in the 1990's. They are in practice based on an 20 year old reference male whose statistics rely on data gathered between the nineteen fifties and seventies. To apply these assumptions to a non homogenous population would ignore many changes. In view of this a significant effort to collate and present data in this area has been made and is available in Malone et al.,(1990 a, b),WHO,(1990). A summary of some of the observations found include:

(a) Dietary Iodine Patterns - Gland mass, uptake and iodine kinetics are highly susceptible to influences of dietary iodine. Adult thyroid mass is routinely 10-12g in iodine rich areas. This is about half the reference value. It increases to as high as 80g in areas where there is an iodine deficiency in the diet. Uptake and clearance times are also very susceptible to influences by dietary iodine and range from 15 to 20 per cent up to almost 100 per cent.

(b) Age and Sex - Significant differences in thyroid characteristics are

noted with age. Mass varies from mq's in the foetal thyroid to 10's of grams in adult thyroids. Uptake also varies being as high as 70% in the case of premature or newborn and rapidly declines to the adult value. Residence time also increases with age with the effective half life in the foetus being approximately half that of the adult. Women have a somewhat different pattern of thyroid metabolism from men.

(c) Medication - Some forms of medication will lead to variation in uptake, e.g. iodine contrast media, and drugs for cardiac and psychiatric uses.

3.7. FOETAL - NEUROLOGICAL DOSIMETRY.

3.7.1. Approximately 1-2% of any population at any time is pregnant. Thus because of the vunerability of this age group special attention needs to be paid to proper dose estimation. However the knowledge of iodine kinetics and thyroid mass in the foetus is lacking. In view of this a systematic review was undertaken from which dose estimates to the foetus were derived (Malone and Gilligan, 1990). The assumption used vary from author to author, and some find the foetal thyroid dose is greater than that to the mother per unit maternal ingestion. Others find that it is about half that to the mother. Arising from this the ICRP recommend the use of the maternal thyroid dose for that of the foetus (ICRP 53, 1988).

3.7.2. As mental retardation is thought to be observed in children who have been exposed in utero to neurological doses as low as 0.04Gy in week eight to sixteen of pregnancy, the dose burden the foetal thyroid might present to the foetal brain upon injestion of 131-I by the mother,was investigated. The necessary foetal anatomical data were derived from the literature and the dose then calculated using standard physical techniques and the uptake/ kinetic data employed in 3.7.1. above. The results for 3 sets of biokinetic data are presented in Fig. 7 (b), which demonstrates the mean neurological doses are 5 to 6 orders of magnitude less than the thyroid doses. In view of this the material and foetal thyroid doses would need to be sufficiently large to cause ablation before direct neurological radiation damage might be anticipated (Gilligan and Malone, 1990).

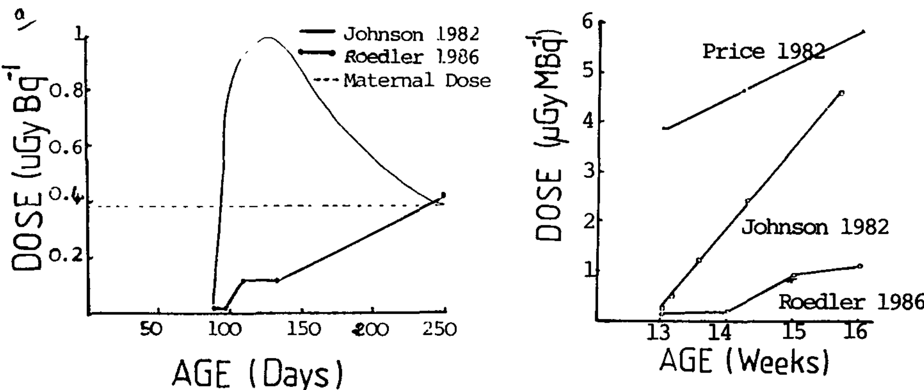


Fig. 7: (a) Comparison of two estimates of foetal thyroid dose to maternal thyroid dose per unit I-131 ingestion as a function of age.

(b) Dose to foetal thyroid from foetal brain per unit I-131 ingestion as a function of age.

3.8. ABSORBED DOSE AS AN INDEX OF HARM.

Absorbed dose estimates are frequently used in association with various Risk Coefficients to evaluate the overall harm that arises from thyroid irradiation The question of whether this is the most appropriate parameter to use, or not, must be reviewed. For example, it is not unreasonable to assume that there is an approximate relationship between cell number and mass in the thyroid. In the absence of data to the contrary it is also not unreasonable to assume that the rate of transformation per cell per unit dose is the same in small and large glands. The consequence of these two simple assumptions is that absorbed dose is not the appropriate parameter to use in derivation of risk estimates. For example 20g and 80g glands both receiving identical absorbed doses would have risk estimates that are different by a factor of four. On the other hand if both glands took up identical amounts of activity this would give rise to identical risks even though the absorbed dose in each case is different. The latter conclusion relies on the traditionally assumed linearity of risk estimates with dose.

The above considerations indicate that dose is not the appropriate physical parameter to associate with risk estimates. The total activity taken up or total energy deposited may be more suitable, and indeed the latter has been suggested for similar purposes elsewhere (Malone et al., 1990 a,b).

3.9. MICRODOSIMETRIC METHODOLOGY.

Dosimetric studies were undertaken both in terms of development of automated methods of analysis of the geometrical properties of cells/ follicles in the cultures, and in terms of refinement of the models for calculation of dose to the cultures on a microscopic scale. A positive feasibility study of the former indicated that it would be possible to use an image analysis system to store the geometric information required for detailed dose calculations at the cell/follicle level. The system uses a microscope coupled to a video camera which in turn was interfaced to a nuclear medicine image anlaysis computer using a frame grabber. In practice calculations at this level of detail have not been needed to date as only 131-I has been used, as no nuclides with very short range beta particles were employed. However microdosimetry of 131-I has been revitalized by new work now appearing on the dosimetry labelled antibodies targeted at microfoci of tumours.

3.10. ASSAY FOR INDICES OF CELL TRANSFORMATION.

3.10.1. The biological assessment systems available in this project all involved cell/tissue cultures. Apart from practical or ethical considerations this was for two main reasons. First it eliminated the uncertainties that had plagued interpretation of in vivo work with radionuclides. Second with respect to cell transformation and related end points it permitted design of experiments that had the potential (at the very least) of contributing to the insight available into the transformation/ dose relationship, without embarking on the so called "megamouse" experiments. Optimism in this area was further enhanced by the fact that relative information on the r.b.e. of 131-I viz a viz high dose rate gamma rays would in itself be valuable even if absolute data on rates of transformation were not available. Some of the indices examined include (i) Senescence/Immortality in Cell Cultures; (ii) Focus Formation in Monolayers; (iii) Growth on Soft Agar; and (iv) LDH Isoenzyme Analysis. Of these (iii) is possibly the most demanding. However each is briefly

of cells to be examined for LDH profile. This has shown that the normal predominance in human thyroid cells of LDH 1-3 with a peak at band 3 is changed and bands 4 and 5 appear in greater quantity as illustrated in Table 3, which is consistent with alteration towards transformation.

3.11. DOSE RESPONSE RELATIONSHIP FOR INDICES OF CELL TRANSFORMATION.

3.11.1. All of the endpoints discussed in 3.10 lend themselves to a certain level of quantitation. However the assay for growth on soft agar lends itself to, perhaps, a slightly more rigorous form of quantitation. Examples of results for sheep thyroid cells are presented in terms of the number of soft agar clones per exposed cell in Table 6 and Fig. 8 below.

3.11.2. A level of caution must be applied to these results for a number of reasons. First while soft agar growth is a reliable and reproducible endpoint, all attempts to form tumours in nude mice by inoculating them with agar positive cells have proven negative. The validity of the nude mice assay as a definitive endpoint, which holds for fibroblast systems is, however, open to question in epithelial systems since epithelial tumour cells tend to terminally differentiate in nude mice.

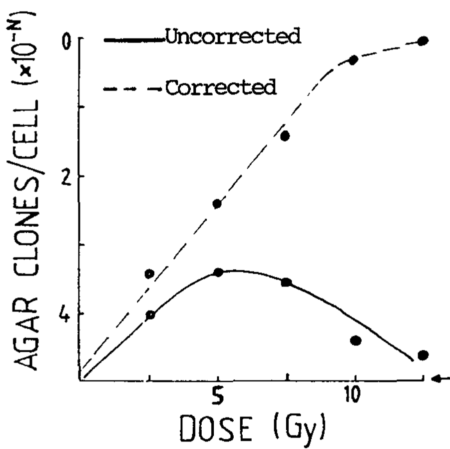


Fig. 8: Number of soft agar clones detected in cultures of sheep thyroid versus dose, upper curve illustrates the effect of considering lethal mutations.

SOFT AGAR POSITIVE CLONES DETECTED IN CULTURES OF SHEEP THYROID (N=10) EXPOSED TO DOSES OF 60-Co RADIATION IN THE RANGE 0 - 12.5 Gy.

TABLE 6.

DOSE	CLONES/CELL EXPOSED	PASSAGES ELAPSING TO SOFT AGAR DETECTION
0	1.1×10^{-5} (Bgr.) for unirradiated cells.	-
2.5	9.9×10^{-5}	12 ± 2
5.0	4×10^{-4}	11 ± 1
7.5	2.7×10^{-4}	7 ± 2
10.0	4.0×10^{-5}	5 ± 1
12.5	2.5×10^{-5}	4 ± 1

discussed, with examples, below.

3.10.2. Senescence measurements/immortality in cell lines: Subculture of samples from irradiated sheep and human cultures was attempted to assess the number of cell divisions which occur before senescence of the controls. Results are illustrated from nine patients. (In some other cases the epithelial cell plating efficiency was too low to allow confluent epithelial cultures to develop and contamination with fibroblasts which can undergo up to 22 passages made interpretation of results impossible). The results in Table 4 show the mean value over 3 patients of the cloning efficiency of human thyroid cells.

TABLE 4: CLONING EFFICIENCIES.

Dose (Gy)	Passage No. 1	2	3	4
0	1.2 ± 0.1	0.4 ± 0.06	0	0
2.5	1.1 ± 0.7	1.0 ± 0.08	1.2 ± 0.1	1.0 ± 0.09
5.0	0.016± 0.001	0.8 ± 0.09	1.0 ± 0.08	1.2 ± 0.1
7.5	0.002± 0.001	0.6 ± 0.03	0.8 ± 0.1	1.0 ± 0.08

It can be seen that senescence occurs early in human thyroid cultures, but where high dose has taken place it was possible to maintain rate irradiation cultures for many passages.

3.10.3. Focus formation on the monolayer is widely used as a marker for alteration of cell status. This occured in both sheep and human cells. A correlation was found between number of foci developing on the monolayer of irradiated human cultures and the radiation dose. This is illustrated in Table 5.

TABLE 5: Focus Formation in Human Thyroid Cell Cultures.

Patient	Original Radiation Dose (Gy) 2.5	5.0	7.5
1	6.3 ± 0.4	10.0 ± 0.8	10.2 ± 0.8
2	1.1 ± 0.2	5.3 ± 0.4	3.2 ± 0.2
3	7.7 ± 0.7	11.2 ± 0.9	15.0 ± 0.3
4	3.4 ± 0.4	10.2 ± 0.8	12.2 ± 1.3

The pattern of focus formation appears to increase up to about 5 Gy and then plateau to 7.5Gy. This is consitent with other indices though the data is subject to much uncertainty.

3.10.4. Soft Agar Growth: This was observed in a reproducible form in all high dose rate gamma ray irradiated sheep thyroid cultures. However it still has not been detected in any of the human thyroid cultures examined. It is possible that some technical modifications are necessary, and in particular it may be necessary to isolate and grow up the cells composing the foci referred to in 3.10.3 above. Further discussion of this area takes place in 3.10.11 and 3.10.12 below.

3.10.5. LDH Isoenzyme Analysis: A shift towards the anaerobic forms in the LDH isoenzyme profile is characteristic of malignant cells in vivo. A microelectronphoresis technique was developed which permited small numbers

Survival here is residual survival calculated by taking the plating efficiency drop at each passage into account as in Mothersill and Seymour (1987b).

Second it has been established that late lethal mutations occur in both sheep and human thyroid cultures and these are manifest only after serial subculture. As an approach to the determination of the effect of this on survival, the C3H 10T½ transformation model was used as its relationship with tumorigenicity in vivo is known. Using the C3H system it is apparent that the observed transformation frequency is profoundly affected by taking lethal mutations into consideration and the dose response curve does not plateau or invert in the range 0-15 Gy. When a correction is applied to the 'bell shaped' curve obtained for soft agar clones from sheep thyroid, the decrease in number of transformed clones at high radiation doses is removed (Fig. 8) even though the validity of this correction remaining to be fully established. The correction is difficult to apply to thyroid culture data because of the problem of senescence of controls which prevents proper control plating efficiencies being established at the passages where cultures show agar positive clones.

3.12. 131-I AND HUMAN INVESTIGATIONS.

3.12.1. The most demanding index of cell transformation used with the cell culture assays described above is the detection of soft agar growth. While this assay has been reliably applied to sheep thyroid cells irradiated with high dose rate irradiation, it has not been possible to produce soft agar positive cells using 131-I. This may indicate an absence of 131-I induced "transformation" or technical failure in the system. Rigourous steps have been taken to exclude the latter in a large number of repeat experiments, but to date no transformation after 131-I has been observed in sheep cells. However it should be noted that there is a significant change in the senescence pattern after 131-I.

3.12.2. Repeated attempts to apply the soft agar assay with human cultures have also produced negative results whether they were irradiated with 60-Co or 131-I. No clear reason for this has emerged over and above the general difficulty noted by most workers in producing transformation with human material. However using alternative endpoints it is noteworthy that Nakamura et al. (1989) have observed mitogenesis in thyroid cell cultures with frequencies not dissimilar to the soft agar growth noted in Table 6 and Fig. 8 above for sheep.

TABLE 7

End Point	Source	r.b.e. for 131-I	Comment
Cancer	UNSCEAR, 1977	1	
	Maxon, 1985	0.3 (0.1 1.0)	Uncertain
	Laird, 1987	0.6 1.0	Dosimetry
	UNSCEAR, 1988	0.3 (0.1 0.5)	
	Holm, 1989	0	
	Hamilton et al. 1989	0	
Neoplasia	Maxon, 1985	0.2 - 1.0	
Proliferative	Malone, 1975 et seq.		
(Rat Thyroid)		0.1	Uncertain
(Human Culture)		0.16 - 0.2	Dosimetry
(Sheep Culture)		0.2	

3.12.3. From the above the amount of information that can be added to the data on r.b.e. for 131-I is relatively little. This is summarized in Table 7 for both proliferative and non proliferative endpoints. However the findings do tend to support the lower rather than the higher values suggested for r.b.e.

3.12.4. The epidemiological and related work in this project was conducted in the main in association with the post Chernobyl initiative and is reported there. However two points are worthy of mention here. First calculation of the expected rate of incidence of neoplasia from the above data in sheep and Nakamura's (1989) human data gives results within one or two orders of magnitude of those expected from risk estimate calculations. This is reasonably encouraging given the conceptual and practical uncertainty in the experimental systems and the significant uncertainty (or indeed inaccuracy) in the ratio of death rate to incidence and the ratio of benign/malignant disease used in the risk estimates. Second the inclusion of non fatal harm as a significant element in the total thyroid detriment was achieved in many projections of risk estimate and in the post Chernobyl calculations, a feature which had been a longterm objective of this project.

4. CONCLUSIONS.

This project has demonstrated that the proliferative radiobiology of the thyroid can be understood within the same framework as that applying to other tissues. Survival, recovery and fractionation response in differentiated cell cultures are within the range observed for many mammalian cell systems. The Do for sheep cells is the order of 2 Gy and that for cells of human origin is in the range 0.5 - 2.0 Gy. The r.b.e. for 131-I is 0.16 - 0.2 and the response to it may be explained on the basis of dose rate. Furthermore the thyroid response has been characterized within the framework of a unified model that integrates cell survival data obtained in cultures; older cell survival data using the gland weight assay; the incidence of post irradiation hypothyroidism; and a postulated cell kinetics structure for the gland.

The report also addressed the problem of population dosimetry given the differences in lifestyle emerging in the years since the adoption of many of the anatomic and physiological data for thyroid dosimetry. Special attention was paid to foetal dosimetry and in particular the possibility that the foetal thyroid might contribute a significant burden to developing foetal neurological tissue. This was regarded as particularly important given the emphasis of radiation induced neurophysiological impairment arising in weeks 8-16 of gestation. The conclusion reached was that maternal and foetal doses due to 131-I ingestion would be similar and that the foetal brain dose would be much less than both by several orders of magnitude. The above investigations were conducted and reported within the framework of conventional thyroid dosimetry. However serious reasons emerged to undermine the appropriateness of absorbed dose as the unit to use in such studies and these are cited in the report.

Studies of Indices of cell transformation yielded interesting results in cultured sheep thyroid cells in which a dose effect relationship was demonstrated for soft agar growth. However "transformation" of this type was not demonstrated after 131-I or with material of human origin. Hence the r.b.e. for 131-I must still remain in some doubt for this area, although it has been clarified for proliferative end points. In summary the projects reported on have added notably to knowledge of thyroid radiation responses and dosimetry during the last four years.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

Prof. M. Cullen, St. James's Hospital, Dublin.
Prof. J.E. Dumont, Hospital Erasme, Brussels.
Dr. P. Smyth, University College, Dublin.
Dr. T.E. Wheldon, Dept. of Clinical Physics and Bioengineering, Glasgow.
Dr. C.B. Seymour and his Wife, St. Luke's Hospital, Dublin.

V. Publications:

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor:

United Kingdom Atomic Energy
Authority UKAEA
11 Charles II Street
GB-London SW1Y 4QP

Contract no.: BI6-D-235-UK

Head(s) of research team(s) [name(s) and address(es)]:

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Title of the research contract:

Consequences to lung and bone of exposure to actinides.

List of projects:

1. Synergistic effects of cigarette smoke in the induction of lung tumors by inhaled $^{239}PuO_2$.

2. Studies on the distribution and effects of alpha and beta emitting radionuclides on the bone and the bone marrow.

Title of the project no.: 1

Synergistic effects of cigarette smoke in the induction of lung tumours by inhaled $^{239}PuO_2$

Head(s) of project:

A Morgan

Scientific staff:

A Morgan
N D Priest
R J Talbot
A Black

I. Objectives of the project:

To demonstrate that exposure to cigarette smoke enhances the incidence of lung tumours in mice previously exposed to $^{239}PuO_2$.

II. Objectives for the reporting period:

To complete the study started under a previous contract (B16-D-100-UK) comparing the incidence of lung tumours in plutonium-exposed CBA/H mice which had received one year's exposure to cigarette smoke with that in animals which were either sham-exposed to smoke or were given no treatment other than their initial exposure to plutonium. To initiate further studies designed to determine the effects of tobacco smoke inhalation by mice on plutonium-induced lung tumours.

III. Progress achieved:

1. Introduction

It is well known that both cigarette smoke and alpha-radiation are lung carcinogens and some studies have shown that when man and animals are exposed to both of these then the numbers of tumours found is larger than would be expected on the basis of a summation of the risk of exposure to each of these carginogens separately (1). This effect is commonly referred to as synergy. Synergy has been demonstrated in rats that were exposed to the products of cigarette smoke after they had been exposed to radon gas and its daughters, although the reverse order of these insults had no such effect (2). These results have been interpreted to indicate that radiation is an initiator of lung cancer and that cigarette smoke is a tumour promoter. However, other experiments have failed to demonstrate synergy and these include a study conducted on radon and cigarette smoke exposed dogs (3). In this experiment the effects of these was found to be antagonistic. it follows, that it is far from clear how these insults interact under different exposure conditions. The present experiments were conducted using plutonium-239 as the source of lung irradiation. Unlike radon and its daughters, plutonium irradiates the lung for a long period after its intake, so that when mice were subsequently exposed to cigarette smoke they received both insults simultaneously. This mimics the most likely exposure regimen for man where exposure to both carcinogens is likely to be chronic and concurrent rather than acute and separated in time. The hypothesis tested was that at levels of exposure to plutonium-239 which have been demonstrated to cause lung tumours in mice, a subsequent chronic exposure to cigarette smoke would increase the tumour frequency. (That is that the effects of tobacco smoke and plutonium in the mouse lung are synergistic). The experiments were conducted on mice which have previously been shown to well tolerate exposure to cigarette smoke (4,5,6).

2. Methods

Three groups of female CBA/H mice were exposed (nose only) to an aerosol containing $^{239}PuO_2$ with an AMAD of 1.5 μm and σg of 1.3. Each animal was approximately 10 weeks of age at the time of inhalation and received an initial alveolar deposit (IAD) of about 100 Bq of ^{239}Pu. At 8 days after inhalation the animals were randomly allocated to one of three treatment groups.

One group, designated PTS, was exposed to tobacco smoke for a period of one year. Each animal received tobacco smoke - from a commercial, UK, middle-tar brand of tipped cigarette - at a concentration of approximately 1.3 mg l^{-1} of tar particulate material for one hour per day, five days each week, for one year. Battelle Mark III smoking machines were used for these exposures and each one hour exposure consisted of two separate half-hour treatments separated by a rest period of 15 minutes. Tests made using cigarettes labelled with iodo(^{123}I)hexadecane, a hydrocarbon with a boiling point equivalent to the mid-point of those of cigarette tars showed that this smoking procedure resulted in an average daily cigarette smoke tar deposit in the lungs of the smoking mice of 92.6±15 μg.

A second group of mice, designated PSS, was sham-exposed according to the protocol described for PTS. The sham exposures were made on a Battelle Mark III smoking machine modified to deliver clean air rather than tobacco smoke.

The third group received no additional treatment and after exposure to plutonium were returned to the animal house as a designated cage control group - PCC.

In addition to the above, two further groups of animals were included in the study. In contrast to the above, none of these animals were exposed to plutonium. One group (OTS) received tobacco smoke, as for the PTS mice, for one year. The other group (OCC) was used as a cage control and the mice in this group received no treatment.

Following the cessation of either sham-smoking or smoking the PTS, OTS and PSS animals were returned to the animal maintenance facility for a further six month period during which time the animals received no further treatment. At the end of this period the mice in all five experimental groups were killed and necropsies performed. For examination, the lungs were inflated with 0.8 ml of formal acetic alcohol fixative. Subsequently, these were excised, dehydrated in alcohol and then cleared in methyl salicyclate. The cleared lungs were examined at low magnification by transmitted light microscopy and any lung lobes containing opacities (mostly tumours) were excised and embedded for histopathological examination. The pathological examination and classification of lung opacities was carried out under contract, in compliance with GLP standards.

In addition to the above, the lungs of some mice from each experimental group were analysed to determine their radionuclide content at death. These were first ashed then scintillation counted using a cocktail comprising a plutonium extractant as well as a scintillant (7).

All the animals were weighed at weekly intervals throughout the experiment and were allowed food and water ad libitum.

3. Results

The mice in the cage control group continued to gain weight during the first year of the experiment. In contrast, those animals which were sham-exposed to smoke gained much less weight - only 4 g compared with 11 g for the caged control animals - and those exposed to cigarette smoke gained practically no weight (about 0.5 g) during this year. At the end of the smoking period the PTS and PSS mice rapidly gained weight, but at the end of the experiment, these remained about 4 g lighter than those animals in the PCC group.

Examination of the lungs of the animals that received plutonium revealed 65 macroscopically defined lesions. Most of these lesions were very small and many would probably have been missed using conventional histopathological techniques. For example, 57% of the lesions were less than 1.5 mm in diameter and 22% were less than 1 mm. The lesions were distributed throughout the lung parenchyma and no association with the major airways was found. The numbers of lesions found in animals exposed to plutonium are shown in Table 1. It can be seen that of the 61 lesions of nodular appearance, 30 were found in the cage-control animals (PCC), 21 in the sham-smoked animals (PSS), but only 10 in the mice which received

tobacco smoke (PTS). A similar trend was found when the percentage of the affected animals in each group were calculated: PCC - 54%, PSS - 51%, PTS - 26%.

The Table also shows the amount of ^{239}Pu remaining in the lungs of the mice in each group at the end of the experiment. The lungs used for the determinations were those of the 74 mice found to contain no macroscopically discernible lesion. It can be seen that the lung contents of the cage-control and sham-exposed mice were very similar, but that the lungs of animals exposed to cigarette smoke contained approximately four times as much plutonium, indicating that the cigarette smoke inhibited the clearance of the radionuclide.

Experimental Group	Number of Mice	Number of Nodules	% with Nodules	Alveolar Bq at Sacrifice
PCC	43	30	53.7%	3.3 Bq
PSS	45	21	51.4%	3.8 Bq
PTS	38	10	26.3%	14.2 Bq

Table 1. Summary of the results of the macrosopic investigation of the numbers of lesions in the cage-control (PCC), sham-exposed (PSS) and tobacco smoke-exposed (PTS) mice.

The results of the histopathological examination of the lungs is summarised in Figure 2. This shows that, excluding 5 metastasis of extra-pulmonary origin, 70 tumours were identified. Of these, 7 were judged to be malignant and 63 benign. Of the benign tumours 20 were solid tumours of alveolar origin showing no tubular differentiation, 32 showed tubular differentiation and were judged to be of bronchiolar origin and 10 were of mixed or uncertain origin. The Table also shows that more than twice as many primary tumours were found in the lungs of the mice in the PCC group as in the equivalent group that were only given cigarette smoke (OTS) and that the number of tumours found in the sham-exposed group (PSS) was intermediate between those of the other groups that received plutonium. A closer examination of the results showed that there was no clear difference between the numbers of tubular-bronchiolar-benign tumours found in each of the experimental groups, but the other tumours types were generally much less prevalent in the lungs of the mice that were exposed to cigarette smoke. With the exception of a single tumour in the PSS group all the malignant tumours were found in the cage control mice (PCC).

The animals which received no plutonium contained fewer tumours.In both the OTS and OCC groups, the tumour incidence was approximately 20%, compared with the PCC animals, the OCC mice contained only $\frac{1}{3}$ as many tumours. However, the difference between the OTS and PTS animal groups was much smaller indicating an inhibitory effect of cigarette smoke on tumour production.

4. Conclusions

As expected, the results of this study show that an IAD of 100 Bq of plutonium-239 results in a significant enhancement of the incidence of lung tumours. However, when the results of the synergy study were examined, a much less expected result was found in that the numbers of lesions in the mice exposed to both plutonium and cigarette smoke was lower than was found in either of the other groups of animals which received plutonium only. This result may be interpreted in two ways. Firstly, the result may be interpreted as indicating that the effects of alpha-particles and cigarette smoke on the lung are antagonistic and that cigarette smoke had a protective rather than synergistic effect on the lungs of the mice. Alternatively, it may be that the promoting effect of cigarette smoke, together with the greater radiation dose to the lungs of the mice which received tobacco smoke - due to impaired clearance of plutonium - may have had a lowering effect on the tumour incidence. This may have arisen if smoking had resulted in increased radiation-sterilisation of transformed cells. Such an explanation is tenable because a dose response study for ^{239}Pu conducted in the same strain of mouse showed that at one year after exposure to plutonium the peak tumour incidence corresponded to an IAD of 160 Bq which is not very much greater than the 100 Bq used for the present studies. Further, more extensive studies are currently being undertaken using lower doses of plutonium and higher doses of tar. These will be completed in 1991.

	Cage control	Sham smoked	Tobacco smoked	Cage control	Tobacco smoked
	-----Plutonium exposed----				
	(PCC)	(PSS)	(PTS)	(OCC)	(OTS)
Solid Alveolar Benign	6	4	3	6	1
Mixed/uncertain Benign	1	3	0	3	3
Mixed/uncertain Malignant	1	0	0	0	0
Tubular Bronchiolar Benign	7	8	7	5	5
Tubular Bronchiolar Malignant	6	1	0	0	0
Metastases Extra-Pulmonary origin	4	1	0	0	0
Total excluding Metastatic deposits	21	16	10	14	9
Total Benign All types	15	15	10	14	9
Number of mice in group	43	45	38	58	46
% Mice with Tumours	46.5%	33.3%	26.3%	18.9%	19.6%
Tumours per Mouse	.58	.38	.26	.19	.20

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IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

None.

V. Publications:

Talbot R.J., Morgan A., Moores S.R. and Matulionis D.H. (1987) Preliminary studies of the interaction of plutonium dioxide and cigarette smoke in the mouse lung. In: Proceedings of EULEP Symposium - Effects after combined exposures to ionizing radiations and chemical substances. Pisa, 19th September 1986. Int. J. Radiat. Biol., 51, 1101-1110.

Priest N.D., Moores, S.R., Black A., Talbot R. and Morgan A. (1989) The combined effects of plutonium and cigarette smoke on the production of lung tumours. Paper presented at 4th Int. Symp. Malvern, June 1989.

Morgan A., Black A., Moores S.R., Nicholls L., Orpwood F., Purbrick W F., Talbot R.J., Walsh M and Matulionis D.H. (1987) Combined effects of exposure to cigarette smoke and $^{239}PuO_2$. 2. Results of a 3-month trial exposure to cigarette smoke only. AERE-R 12337.

Talbot, R.J., Moores S.R., Walsh M., Purbrick W.F. and Knight D. (1986) Co-carcinogenicity of cigarette smoke and plutonium dioxide: 1. Impairment of lung clearance of plutonium-239 dioxide from mice by exposure to cigarette smoke. AERE-R 12098.

Title of the project no.: 2

Studies on the distribution and effects of alpha- and beta-emitting radionuclides on the bone and bone marrow

Head(s) of project:

A Morgan

Scientific staff:

N D Priest
A Morgan
GR Morgan
JP Kellington

I. Objectives of the project:

1. To determine the comparative distribution of plutonium and americium in the bones and bone marrow of baboons, following their administration in different chemical forms by injection and inhalation.

2. To study the effects of bone turnover and growth on the redistribution of plutonium and americium, and the influence of macrophages on their distribution in the red bone marrow.

3. To use the data obtained to construct realistic models for the dosimetry of bone-seeking radionuclides in the human skeleton.

II. Objectives for the reporting period:

- To continue to examine, by autoradiography, the bones of plutonium and americium contaminated baboons and man.

- To undertake a quantitative analysis of the distribution of plutonium in baboon bone at different times after its injection.

- To apply the dosimetric model developed for plutonium in man and to estimate the risks of leukaemia following plutonium intakes.

- To study the appropriateness of risk estimates based on Thorotrast toxicity data.

III. Progress achieved:

During the current year, considerable progress has been made on the validation and development of metabolic models for bone seeking radionuclides. Particular attention has been given to two aspects of our current dosimetry programme.

1. Dosimetric Modelling

The first of these was a comparison of the dosimetry models published by ourselves, by the ICRP and by Leggett at Oak Ridge, USA. The first stage was the conversion of each model to similar and compatible computer codes so that they might be compared. This stage was completed early in the financial year. Subsequently, the models were run for plutonium-239 intakes by adults and by children of different ages. With respect to body retention and the retention half-time of plutonium in the skeleton, very good agreement was found between our model and that of the ICRP. However, the Oak Ridge model was found to be deficient to the point where it was obvious that it had never been run. Consultations with the NRPB revealed that the ICRP were to adopt a revised Leggett model for its age-related dosimetry and this model was also tested. In our opinion the revised model is also flawed, but the ICRP has apparently decided to continue with its use.

The second study area is concerned with the problem of model accuracy. It is self-evident that any dosimetric model is only as good as the data and parameters incorporated within it and the uncertainties in these introduce uncertainties in the doses calculated by the model. In order to obtain an estimate of the accuracy of our model, a full sensitivity analysis has been undertaken. For this analysis the value of all the parameters used for the model are allowed to vary simultaneously according to our knowledge of their uncertainty. Multiple runs of the model were then undertaken in order to ascertain the distribution of its predictions. So far the results indicate that uncertainties in the modelling are likely to be less important for the prediction of consequences than uncertainties in risk estimates. This would seem to be true particularly for leukaemia induced by plutonium and related elements.

2. Actinide Deposition and Redistribution in the Skeleton

The effects of plutonium redistribution in baboon bone, supplied by the CEA, France, on its dosimetry in the skeleton have been investigated. These studies were undertaken to provide information from a near human animal that can be used for the development of metabolically meaningful dosimetric models. The results have been produced by the examination and quantification of autoradiographs of bones from baboons killed at different times after the intravenous injection of a soluble plutonium TBP complex. The results obtained during the period of this project show that, despite substantial bone turnover amounting to more than 100% per year in both cortical and travecular bone, the radiation dose received by bone surfaces is affected to a much smaller extent than expected. For example, at 1 day after injection the fraction of the total alpha dose absorbed by the radiosensitive bone surfaces ranges from 15-20%, compared with an ICRP expectation of 25%; at 1 month bone turnover had reduced this to 10-11% and this level was then maintained so that it remained 7-12% at 1 year post-injection. These results are consistent with the dosimetric assumptions made in our modelling. Similarly, no increase in radiation

dose received by the bone marrow as a consequence of its accumulation in marrow macrophages was found and the fraction of the radiation dose received by this organ remained at about 10%.

Studies on human bone, contaminated with americium-241, have also been undertaken. These use bone removed by us at autopsy from an American male radiation work who died 11 years after a substantial radionuclide intake. The bones were donated by the United States Transuranium Registry and the current studies form part of the Registry's coordinated examination of the cadaver. Qualitatively, the results show that little radionuclide redistribution had occurred during the 11 years in the patella and this bone also showed little radiation damage. Other bones exhibited more bone turnover and consequently greater americium redistribution. In the clavicles, limited americium redistribution was evident and in the ribs more was identified along with evidence of bone growth (drift). Both these bones showed some radiation damage including peritrabecular fibrosis and the initial surface pattern of radionuclide deposition was largely retained indicating the importance of secondary bone surface deposits. In the sternum, radiation damage was more evident and bone surface deposits of americium at many sites had become buried by reactive bone, but in this bone also, the relatively even surface pattern of deposition remained. However, in the lumbar vertebrae massive radionuclide redistribution was evident and many bone surfaces were depleted in americium. Only the surfaces of bone trabeculae towards the centre of the vertebral body seemed to retain their initial americium burden. In all bones, only a few percent or less of americium was present in the red bone marrow and none was present in the yellow bone marrow of the patella. Overall, the behaviour of actinide in the human bone was very little different from that seen in similarly contaminated dog and monkey bone emphasising the importance of the baboon studies. As yet, no quantitative assessment of the human bone has been made.

3. Validity of Current Risk Estimates

Volunteer studies on the metabolism of the radium-like alkaline earth element barium are continuing. These were initiated in order to check the validity of current estimates of the dosimetry of radium in man. This is important because our knowledge of the toxicity of radium-226 provides the basis for estimates of the risks associated with intakes of all alpha-emitting, bone-seeking radionuclides. The experiments were initiated over 20 years ago. To date, six male volunteers have been injected with the long-lived radioisotope barium-133. Of these, five have been injected for at least two years and the sixth was injected during 1989. The results show that, while the initial uptake of barium by the skeleton is variable, its clearance rate during the first few years post-injection is remarkably uniform. Clearance during this period is mostly by heterionic exchange. Substantial clearance data at later times is currently only available for one volunteer. This man was injected at age 61 and then again at age 81. The data for this individual suggest that the long-term clearance is dominated by bone resorption and this appears to be significantly enhanced at the older age. This may be important as most of the human radium data is for women and they have a higher rate of bone loss in later life than men. In addition to the above, studies on the local rates of barium loss from different parts of the skeleton are continuing. These have demonstrated differences in the rate of bone

turnover in different bones. The data collected will be considered for use in advanced metabolic models for osteophilic radionuclides.

It is known that following intakes of plutonium and other bone-seeking radionuclides, the bone marrow is irradiated by plutonium present both on bone surfaces and in the bone marrow. It is also known that current risk estimates as recommended for use by the ICRP cannot be confirmed by reference to studies of human populations, all of which indicate very much lower risk estimates. Currently, we are examining experimentally the validity of the current human toxicity data bases to see if there are any contra-indications to their use for the prediction of leukaemia in human populations exposed to plutonium. Previous studies with pig bone have shown that radium isotopes become deposited in the skeleton in much the same way as the actinides and that the absence of substantial excess leukaemia in human populations exposed to radium-224 must therefore be taken to indicate that the risk of leukaemia in people exposed to actinides is also low. However, actinides, but not radium, are accumulated in the bone marrow. Accordingly, we are currently studying the toxicity data for Thorotrast which is deposited in and irradiates the bone marrow with alpha-particles. Two aspects are being examined, firstly the uniformity of Thorotrast deposition in the bone marrow at different skeletal sites and secondly, how much Thorotrast is deposited in the red bone marrow as opposed to the fatty, yellow bone marrow. If it is found that Thorotrast is heavily concentrated in some parts of the red bone marrow but absent from others, then by invoking the concept of tissue sterilisation it may be possible to explain why so few leukaemias are seen in Thorotrast patients. Tissues from monkeys injected with Thorotrast and from a human who died from aleukaemic leukaemia as a consequence of her Thorotrast intake (supplied by the United States Transuranium Registry) have been used for the study. The human tissues were collected at autopsy. Techniques being employed initially included back-scattered electron imaging using the SEM and secondary ion mass spectroscopy (SIMS). These yielded excellent qualitative and locally quantitative results showing that some macrophages in the red bone marrow comprised 30% of thorium by mass, but the comparison of different sites proved problematic. It was therefore necessary to seek alternative methods for this task. As a result we were able to show that very good results could be obtained using X-ray fluorescence (XRF) in combination with limited image analysis. Results are now being collected from monkey bone removed one day after the injection of Thorotrast. Currently it would seem that the concentrations of thorium in the red bone marrow are in the order of 120 $\mu g\ g^{-1}$ and are about 10 times lower in the yellow marrow. Other monkeys which were injected with Thorotrast over 1 year ago remain alive. Complete tissue analyses are being undertaken. To data no results have been obtained which would suggest that there is any special feature of Thorotrast distribution which could explain its low toxicity.

4. Effect of LET

In addition to the above, the programme of experimental work designed to compare the effects of low and high LET radiations on the bone marrow has continued. This uses injected radiolabelled fused clay particles (FAP) as the vector for delivering the radiation dose to the bone marrow and other tissues comprising the reticulo-endothelial system. The system for the production of radiolabelled fused clay (FAP) is now fully installed and

particles labelled with cobalt-57 have been produced for use in preliminary studies. Curium-242 is currently being produced by the Chemistry Division, Harwell, and will be available for use by the end of March 1990. During the current year, studies have been carried out to determine the distribution and retention characteristics of FAP following its injection into mice and leaching experiments have been completed to measure the *in vivo* and *in vitro* dissolution rates of the material. In a preliminary study it was found that a large amount of the injected FAP was retained at the injection site. This was reduced to less than 5% in later mice which were injected by an improved technique. In these animals 80-90% of the clay was deposited in the liver with 2-3% in the bone marrow. No difference was found between the deposition patterns of 1 and 3 μm diameter particles. Similarly, both sized particles were found to be essentially insoluble as determined by analysis of excreta. In addition to the above, cytochemical techniques required for the categorisation of myeloid leukaemia according to the FAB system of pathology have been developed in the laboratory and a protocol for the toxicity studies has been written.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

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United States Transuranium Registry
Hanford Environmental Health Foundation
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Dr Robert Schlenker
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FRANCE

V. Publications:

The Bone Volume Effect on the Dosimetry of plutonium-239 and americium-241 in the skeleton of man and monkeys.
Priest N.D., Haines J.W., Humphreys J.A.M., Métivier H. and Kathren R.L.
In: Proceedings of 3rd International Conference on Low Level Measurements of Actinides and Long-lived Radionuclides in Biological and Environmental samples. Bombay, India, 29 January-2 February 1990.

Chapter 5. The distribution and behaviour of metals in the skeleton and body: Studies with Radionuclides. N.D. Priest.
In: Trace Metals and Fluoride in Bones and Teeth. Eds. Priest N.D. and Van de Vyver F. CRC Press: Boca Raton.

The metabolism and dosimetry of plutonium. N.D. Priest. Forum on alpha-emitters in bone and leukaemia, Committee on the Effects of Ionizing Radiation, London, November 1986.

Sensitivity Testing of an Age-related Multicompartment Dosimetric Model for Bone Surface-seeking Radionuclides in Man. N.D. Priest and A. Birchall. Health Physics (In press).

Alpha-emitters in the skeleton: an evaluation of the risk of leukaemia following intakes of plutonium-239. N.D. Priest. British Institute of Radiology (BIR) Report 21, p 159-166 (1989).

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: B16-D-092-IRL

St. Luke's Hospital
Highfield Road
Rathgar
IRL-Dublin 6

Head(s) of research team(s) [name(s) and address(es)]:

Dr. C. Mothersill
St. Luke's Hospital
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IRL-Dublin 6

Telephone number: 01-974552

Title of the research contract:

Inter-related studies on dose dependence and mechanisms of radiation induced carcinogenesis and environmentally induced and radiation promoted carcinogenesis.

List of projects:

1. Radiation transformation in primary culture systems.

Title of the project no.: B16-D-092 IRL

Head(s) of project: Dr C Mothersill
Dr C Seymour

Scientific staff: Dr C Mothersill
Dr C Seymour
Ms A Cusack
Ms M McDonnell
Ms P Nolan
Mr V Rodilla
Mr C Grimes

I. Objectives of the project:

1. To develop methods for culture of tissue of interest in the radiation protection field.
2. To study the induction by radiation of features associated with carcinogenesis in these culture systems.
3. To investigate mechanisms involved in the development of selected radiation transformation endpoints in the culture systems.
4. To attempt to quantify radiation transformation frequencies in selected primary culture systems.
5. To investigate the potentiating effect of known tumour promotors such as phorbol esters.

II. Objectives for the reporting period:

1. To integrate immunocytochemical and ultrastructural analysis of irradiated tissues in an attempt to quantify cellular changes.
2. To develop a mathematical model for radiation induced transformation expression which incorporates a lethal mutation term.
3. To continue to develop 3-D culture systems for available tissue.
4. To quantify the biochemical changes detected in isoenzymes and intermediate filaments on a per Gray cell basis following irradiation.

III. Progress achieved:

INTRODUCTION

Carcinomas account for 92% of tumours diagnosed yet research papers dealing with epithelial cell transformation only composed around 8% of the literature on the subject in the last five years. The situation regarding radiation transformation research is worse, with less than 0.05% of papers dealing with radiation transformation of epithelial cells. Tentative conclusions which may be drawn from this are that (a) we are looking at the wrong cells in the wrong way and (b) we cannot look at the right cells.

The argument is often put forward that general information may be obtained from less than perfect models and extrapolated or used as a basis for the deduction of relevant answers. In the case of carcinogenesis this argument is hard to justify. Epithelial cells from which carcinomas arise are usually differentiated, highly specialised and functional with a polarity and precisely determined position in a tissue structure. In the human, tumours usually arise late in genetically dissimilar individuals after the tissue has functioned normally for maybe 60-70 years. While extrapolating from the simple to the complex may be a useful scientific method, there is a grave danger of the work becoming irrelevant when the real situation in which cancer arises is so far removed from the model used. The ideal model should be human, epithelial, three-dimensional, differentiated and with quantifiable and definitive endpoints. Many scientists have attempted to develop human epithelial systems have met with variable degrees of success. Among them are DiPaolo, who has used virus transfection to immortalise human skin and cervical epithelial cells. Mulcahy, who developed a fat pad assay where cells treated in vitro are injected into the fat pad of the rat, surviving clonogens of human cells are then tested for tumorgenicity in immunosuppressed mice; Grafstrom who has developed a system where clonal growth in culture of human bronchial and buccal cells can be achieved and changes monitored following exposure to chemical carcinogens; Seymour & Mothersill who developed a human thyroid system where a high degree of differentiation can be achieved and clonal growth after serial subculture detected in irradiated cells which have an extended lifespan.

Reznikoff has developed a system for the study of long-term neoplastic change in urogenital cells following initiation by virus transfection. None of these systems has yet shown conclusive transformation by radiation. The question arises that, since we cannot detect transformation even

using the most carinogenic doses of radiation, are we looking at the right endpoints or at the problem in the right way? Possible reasons why radiation transformation has not been detected include the following:

1. The event is extremely rare in human cells and the fragments of normal human tissue available do not supply enough cells to give a statistical chance of detecting a transformant;
2. Cooperation of two or more cells or cell types is required - it is known that stromal elements are important, vascular elements probably are as well.
3. Differentiated function may be required. This latter suggestion is attractive if the translocation theory of cancer induction is favoured since the activator gene sequence controlling some differentiated function in the normal cell and now lying next to the oncogene in the translocated chromosome would need to be switched on for the theory to work.

Because of the complexity of the problem of epithelial cell carcinogenesis, this project approached the problem in the following manner:

1. Only human tissues were used for development of the system.
2. Cells were treated as soon as the tissue explants had attached in culture so that the structural integrity of the tissue was maintained during radiation exposure.
3. After treatment the behaviour of <u>epithelial</u> cells within a <u>mixed</u> culture was monitored.
4. Conditions which favoured differentiation rather than growth of the cells were created.
5. Endpoints were flexible and what does rather than what should happen was documented.

<u>METHODS</u>

1. <u>Sources of tissue and Development of the culture system</u>:

Sources of normal human tissue in useful quantities are scarce and therefore the choice of tissue was limited by availability although efforts were made to choose radiation sensitive organs, or tissues of particular interest in radiation protection studies. Regular supplies of the following normal tissues have been secured; Ureter from transplant donors, cervix, endometrium and ovary from hysterectomy patients, oesophagus from patients undergoing surgery for adenocarcinoma of the stomach, thyroid from patients undergoing pharyngolaryngooesophagectomy for

neoplasms of the upper oesophagus and skin from many different operations. Occasional samples of normal breast tissue have also been obtained. The tissues from tumour bearing patients were used with caution and only where no other source of normal human tissue was available.

All tissues were set up as primary explant cultures. Thyroids were also digested and set up as primary cultures according to the methods developed by the group in earlier contracts. Relevant tumours were used as positive controls.

2. Development of Assays for monitoring radiation induced changes:

The small amounts of normal tissue available and the relatively low numbers of cell divisions undergone by normal cells prior to terminal differentiation, meant that methods for detecting early changes in irradiated cultures had to be developed for use with extremely low numbers of cells. Emphasis was therefore placed on developing in situ techniques (hybridisation, immunocytochemistry, and autoradiography). These methods had the added advantage that they did not require the structure of the culture to be destroyed and results could be interpreted in the context of the culture histology, ultrastructure and degree of differentiation.

3. Irradiation:

Cultures were usually irradiated using a standard cobalt 60 teletherapy unit although some thyroid cultures were exposed to ^{131}Iodine and preliminary experiments with ^{32}P were undertaken. Cultures were generally irradiated when the explants had attached (24-36 hrs after explantation) but before outgrowth had commenced so that the cells were irradiated under conditions where the tissue architecture was as close to the in vivo situation as possible. Post irradiation cultures were stored under conditions which did not especially favour normal cell growth but which would allow the build up of growth factors or angiogenesis factors should these processes be initiated by the treatment.

Results:

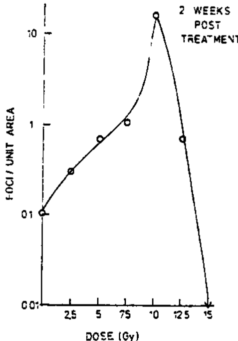
1. Culture system

With all tissues tested the culture method used produces normal, epithelial cells which have been shown in the case

of the oesophagus and ureter at least to be similar in structure to cells in the parent tissue. The cultures proliferate for 2-4 weeks and then undergo terminal differentiation unless treated. Stromal elements, while present, do not dominate the culture. Endothelial cells organise over the epithelial monolayer and form capillary-like tubes. Stratification of squamous epithelium is detectable after 3-4 weeks of culture.

2. Changes in proliferation following irradiation

Following irradiation of the culture the overall outgrowth area is reduced in a dose-dependent manner and the numbers of proliferating cells detected by autoradiography is also reduced (to 10% at 5 Gy). However, certain areas of the culture show proliferative foci which are strongly positive when tested using the proliferating cell antibody Ki67. The numbers of foci increase with radiation dose up to 5-10 Gy and then decline (Fig. 1(a)).



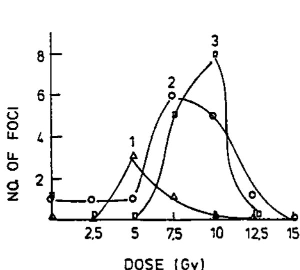


Fig 1 Numbers of proliferative foci detected at increasing radiation doses (1a) over 1-3 weeks (1b)

The dose causing peak numbers of proliferating foci in the culture varies from 5Gy after one week in culture to 10 Gy after three weeks in culture (Fig 1(b)). The nature of the cells composing these foci is not known but their ultrastructure has features similar to those of highly differentiated squamous carcinoma cells, in particular the characteristic ring of tonofibrils around a nucleus pitted with nuclear pores.

Estimates of the rate of "repopulation" or rate of growth of all survivors in the culture after different doses of radiation may be obtained by determining the cell number or

number of proliferating cells at regular intervals. This type of data shows clearly (Fig.2) that after high doses of radiation the growth rate of survivors is faster than after low doses although the final total number of cells in the culture is almost the same.

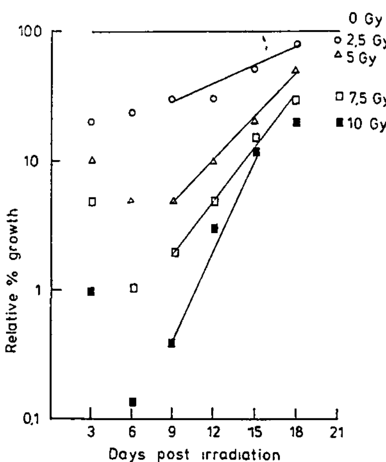


Fig 2 Relative numbers of cells in the outgrowth with time after irradiation, controls normalised to 100% for each time point

3. Cytokeratin profile

The range of cytokeratins occurring in normal, normal irradiated and tumour oesophageal cells in culture is shown in Table 1. Differences between the normal and the other two groups, particularly in the occurrence of cytokeratins 18 and 19, are apparent.

TABLE 1

Sample	Dose	Keratins present
Normal control *in vitro*	-	4,5,6,8,14
Tumour " " "	-	4,5,6,8,14,18,19
Normal control *in vivo*	-	4,5,6,8,14
Tumour " " "	-	4,5,6,8,14,18
Normal *in vitro*	2.5 Gy	4,5,6,8,14,18,19
" " "	5.0 Gy	4,5,6,8,14,18
" " "	10.0 Gy	4,5,6,8,14,15,18,19
" " "	15.0 Gy	4,5,6,8,14,15,18,19
" " "	Nitrosamine + 5 Gy	4,5,6,8,14,18,19
" " "	Nitrosamine only	4,5,6,8,14

4. Lactate dehydrogenase isoenzyme profile

A shift in the LHD isoenzyme profile towards the anaerobic forms of the enzyme has been detected in thyroid cells. This was also found in oesophageal cells but has not yet been quantified.

5. Ultrastructure

Various ultrastructural changes have been quantified. Fig.3(a) shows the pattern of occurrence of nuclear irregularities with radiation dose in human oesophageal epithelial cells and Fig. 3b shows how the numbers of intact mitochondria vary with dose. In both cases the damage reaches a peak at doses associated with expression of high absolute transformation frequencies and falls off or stays constant at higher doses.

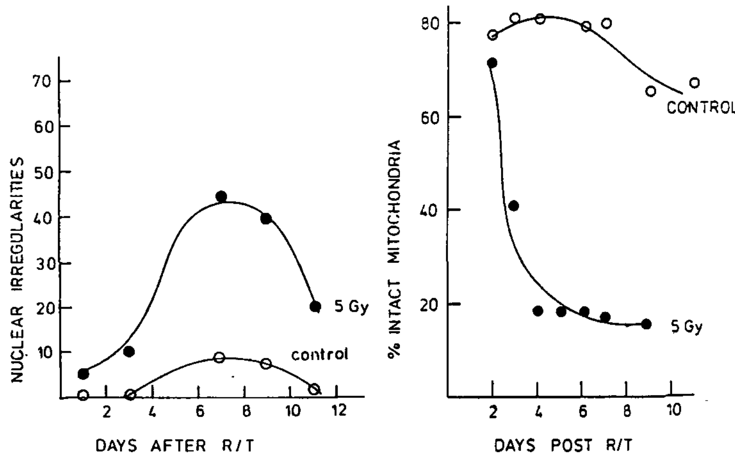


Fig 3 Ultrastructural appearance of cultures exposed to increasing radiation doses. 3a Nuclear irregularities, 3b Mitochondrial damage

6. Endothelial cell proliferation

The relative frequency and absolute numbers of endothelial cells together with their degree of differentiation are shown with respect to radiation dose in Table 2. It can be seen that there is a marked increase in endothelial cell number, frequency and differentiation with increasing dose which peaks 7.5-10.0 Gy.

7. Oncogene Activation

Antibodies to cMyc and nMyc were obtained from Dr G Evan, ICRF, and these revealed cMyc activation in positive tumour controls and in urothelial and oesophageal cultures exposed to 5 Gy gamma rays. This may, however, merely indicate that growth of cell is switched on post irradiation to enable repopulation of the tissue.

TABLE 2

RELATIVE FREQUENCY OF ENDOTHELIAL CELLS IN OUTGROWTHS. COUNTS ARE THE MEAN OF TWO TRANSECTS OF THE OUTGROWTH (EXCLUDING EXPLANT)

DOSE (GY)	TOTAL CELLS IN TRANSECT	ENDOTHELIAL CELLS	% ENDOTHELIAL CELLS	DEGREE OF DIFFERENTIATION	AREA (MM^2)
0	510	8	0.2	None	352
2.5	200	32	16.0	None - slight	80
5.0	61	15	24.6	+	48
7.5	49	36	73.5	+++	40
10.0	34	15	44.1	+++	8
12.5	26	16	61.5	++	3.2
15.0	5	0	0	None	1.6

8. Soft Agar Growth

This endpoint is of dubious use in epithelial cell transformation but as it is widely used for monitoring responses of possibly transformed normal cells and of tumours it was used in occasional experiments where the cell number in the culture made this possible. The test worked best with cultures of thyroid cells. These survived a range of doses where it was possible to quantify the frequency of soft agar colony formation in late passages of the radiation survivors with respect to radiation dose to the primary culture. The relationship was exponential with frequencies of 10^{-3} transformants per surviving cell being obtained 17 passages after exposure to 7.5 Gy. No plateau was reached and extrapolation of the relationship gave a theoretical dose for 10 transformants per surviving cell. Using the explant system it proved too difficult to obtain sufficient cells to perform soft agar assays although cultures of focal cells have now been shown to have greatly extended lifespans and such experiments may be possible with these in the future.

9. Lethal Mutations and Quantification of Transformation Frequencies

During the contract period the failure of survivors of irradiation to divide normally was noticed. Since transformation endpoints are traditionally expressed "per surviving cell" it became apparent that the criterion for judging a cell to have needed to be critically examined. The effect of taking lethal mutations into consideration using the C3H10T system and the thyroid soft agar assay was analysed. The frequency of transformation per surviving cell would seem to be underestimated at high doses using the conventional survival assay.

This problem makes quantification of endpoints with respect of number of survivors in the explant system very difficult and it may be necessary to adopt a different approach to determining quantitative dose/response relationships, for example number or frequency of positive cells at a series of time points after irradiation.

DISCUSSION

One of the major problems in the field of radiation protection is concern over induction of cancers in exposed humans. In order to study the carcinogenic effects of radiation in a systematic way it is clearly necessary to develop experimental systems which will allow the various stages of the carcinogenic process to be produced and quantified in a controlled manner. Ideally such systems should be based on human cells exposed to relevant doses of radiation which then undergo one or more definitive changes which can be quantified with respect to total dose, dose rate, radiation quality, etc.

The results presented are an attempt to develop realistic and meaningful endpoints for human exposure to radiation. A useful endpoint must be reliable, relatively easy to study and given the problems of human tissue supply, should not require large numbers of cells. Because of the inability, to date, of any group to produce definitive, validated transformation of human epithelial cells using radiation, it is also necessary to consider alternative approaches to the qualitative and quantative demonstration of transformation or partial transformation in these cells.

The endpoints found during this contract all show a dose response and are all associated in some way either with transformation to the neoplastic state in vivo or with existing tumours. While none of them in itself is proof of

transformation, taken together they provide evidence of alterations in several biochemical pathways or cellular substructures which change during *in vivo* carcinogenesis. The most promising approach to the problem from a radiation protection point of view would seem to be to assign a weighted value to each of the endpoints and determine a potential transforming factor (PTF) for the tissue for each radiation parameter of interest. This approach will be used in the future.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

V. Publications:

Mothersill, C., Seymour, C.B., Malone, J.F., Moriarty, M.J., Byrne, P. and Hennessy, T.P.J. (1986). Radiation transformation in differentiated human cells in culture. Brit. J. Cancer, 53, Suppl.VII, 251-52.

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Mothersill, C., Seymour, C.B. and Moriarty, M. (1989). Development of radiation transformation systems for epithelial cells - problems and perspectives. Chap. 37 in: Molecular Biology and Cell Transformation, Part 8 of 'Low Dose Radiation' K.F. Baverstock & J.W Stather, eds). London, New York, Philadelphia: Taylor & Francis, pp. 414-422.

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Seymour, Colin and Mothersill Carmel (1989). Are lethal mutations relevant to the calculation of radiation transformation frequencies? In: 'Cell Transformation and Radiation-induced Cancer'. Edited by K.H Chadwick, C. Seymour and B.Barnhart. Bristol and New York: Adam Hilger, pp. 371-385.

ABSTRACTS/PRESENTATIONS TO MEETINGS

Mothersill, C., Seymour, C.B. and Moriarty, M. (1986). Alteration towards transformation in human thyroid epithelial cells in culture. Europ. J. Cancer, Abstract.

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Mothersill, Carmel and Seymour, C.B. Evaluation of the radiation response of normal tissue _in vitro_. British Institute of Radiology Work in Progress Meeting.

Seymour, C.B. and Mothersill, C. Are lethal mutations involved in the development of late radiation effects? International Congress of Radiology, Paris.

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Seymour, C.B. and Mothersill, C. Are lethal mutations relevant to radiation transformation studies? Proc. CEC/DOE/NEB Workshop on Cell Transformation Systems Relevant to Radiation Induced Cancer in Man, Dublin.

Mothersill, C., Seymour, C.B., Bonnar, J. and Moriarty, M. Evaluation of a technique for the prediction of patient response to therapy for gynaecological cancer. Irish Association for Cancer Research, Dublin.

BOOK CHAPTERS/INVITED REVIEWS

Seymour, C.B. and Mothersill, Carmel (1988). Radiation transformation studies: Are they relevant to radiation protection problems? _J. Radiol. Prot., 8_ (3), 129-38.

Seymour, C. and Mothersill, C. (1989). Lethal mutations, split dose recovery and the survival curve shoulder _IJRB_, 56(6), 999-1010.

REPORTS AND THESES

Mulgrew, R.S. (1988). Development of methodologies for determining radiation survival in human cells. MSc Thesis, University of Dublin.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-101-F

Institut de Protection et de
Sûreté Nucléaire
CEN de Fontenay-aux-Roses
B.P. n° 6
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Head(s) of research team(s) [name(s) and address(es)]:

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Telephone number: 1-4654 70 80

Title of the research contract:

Lung modelling contribution: deposition and clearance studies in man.

List of projects:

1. Experimental studies and modelling of deposition and retention of inhaled aerosols in man.

Title of the project no.:

EXPERIMENTAL STUDIES AND MODELLING OF DEPOSITION AND RETENTION OF INHALED AEROSOLS IN MAN

Head(s) of project:

Docteur N. PARMENTIER

Scientific staff:

M. ROY, C.E.A. engineer, with the collaboration of M.H. BECQUEMIN and A. BOUCHIKHI, medical doctors. Respiratory Function Exploration Department Pitié-Salpétrière Hospital PARIS.

I. Objectives of the project:

The development of dosimetric modelling of the respiratory tract for radiation protection is under revision by the International Commission for Radiological Protection. The revised model will use recent knowledge in deposition and retention of inhaled aerosols, in humans and experimental animals. Modelling should apply to all members of the population and therefore consider the dependence of the parameters upon age and the influence of diseases. The proposed work intended to contribute in assessing the important role of the dimensions and of the physiological state of the airways in airborne particle deposition. Such data exist for healthy adults but are missing for children, and rare for patients with impaired lung function.

II. Objectives for the reporting period:

Inhalation of inert insoluble particles was proposed to human volunteers, to measure either total or nasal airway deposition during controlled breathing. Healthy adults and children were to undertake the tests because the changing with age of the respiratory tract geometry and of the ventilation, is usually thought to result in changes of particle deposition values. An accurate estimate of particle deposition in the growing airways is of special interest not only for dosimetry in children, but also for a reliable assessment of accumulated doses during a whole lifespan. Adult patients with impaired lung function were also to be studied for total airway deposition.

The work arose from the international cooperation of aerosol physicists and medical research teams ; it was to be discussed with relevant specialists and the experimental data compared with their calculated airway deposition models.

III. Progress achieved:

METHODOLOGY

Deposition of monodisperse aerosols has been measured in the whole airways, or the nose only, by comparing inhaled and exhaled aerosol concentrations, measured by laser velocimetry. The ventilation was controlled, and the tidal volumes (VT) and frequencies (fR), selected according to age, body size, and sex, in order to standardize the physiological parameters at rest, and under light exercise.

The children values were scaled to the adults' after GAULTIER et al. (1981), GODFREY et al. (1971), and GODFREY (1973), (Table 1).

The aerosol generation-exposure and measurement apparatus (Figure 1) consisted of the following elements :

- a Tri-Jet aerosol generator T.S.I. provided dried aerosols from an aqueous suspension of monodispersed polystyrene beads from Prolabo-Rhône-Poulenc. Three different aerosol outlets could generate simultaneously different sizes of particles : 1, 2.05, 2.8 and 3.1 µm of mass median aerodynamic diameter, (M.M.A.D.) ;

- a Krypton 85 source ionized the air in an adjoining chamber, where the aerosol was electrically discharged (Boltzmann equilibrium) ;

- a diluting chamber mixed the aerosol with an appropriate volume of clean filtered air ;

- an original inhalation device provided subject interface and was made up of ; a Fleisch pneumotachograph n° 2, with a membrane pressure-transducer (Enertec CH 51 +/- 2 mb), measuring inspiratory flow-rates and inspired volume when connected to a flow-rate integrator, to an oscilloscope, for visual monitoring, and to a recorder.

Calibration with a sinusoidal pump permitted display and recording of the inspired volume (Vi) and inhalation time (Ti) values during the subjects' breathing, that was controlled also by auditive frequency signals.

Table 1

Ventilation Parameters Selected to Simulate Two Levels of Activity and Scaled to Age

Age Y	Rest fR (min^{-1})	Rest V_T (l)	Rest V E ($l.min^{-1}$)	Light exercise fR (min^{-1})	Light exercise V_T (l)	Light exercise VE ($l.min^{-1}$)
6	23	0.250	5.75	38	0.290	II.0
8	23	0.280	6.0	37	0.460	17.0
10	20	0.340	6.80	32	0.580	18.6
12 M	18	0.430	7.75	27	0.760	20.5
12 F	19	0.340	6.45	30	0.630	18.9
14 M	15	0.540	8.1	25	0.930	23.2
14 F	18	0.370	6.7	26	0.770	20
16 M	14	0.620	8.7	23	1.10	25.3
16 F	17	0.410	6.9	24	0.900	21.6
18 M	13	0.770	10.0	21	1.25	26.2
18 F	15	0.470	7.05	22	1.0	22
30 M	12	0.750	9.0	20	1.25	25
30 F	14	0.460	6.45	21	1.0	21

fR : frequency
V_T : tidal volume
VE : minute ventilation

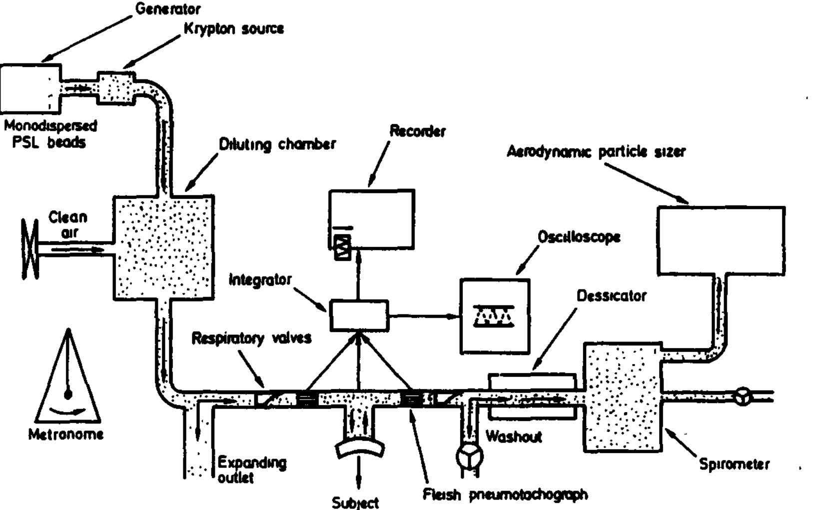


Fig. 1 : **Aerosol generation-exposure system**

Two original respiratory valves (EUGENE et al., 1987) resembling cardiac valvulae and adaptated to large airflows, separated inhaled from exhaled gases with good airtightness and low particle trapping :

- a dessicator trapped exhaled air humidity ;
- a dry spirometer accumulated up to 10 l. of expired gases.

When saturated, the system was automatically disconnected and the contents of the spirometer were aspirated into the particle counter at a constant flow rate of 5 $l.min^{-1}$:

- an aerodynamic particle sizer (A.P.S. 33, T.S.I.) measured the time taken by a particle between the two passages in two laser beams, after a constant acceleration in an airflow of 15 $m.s^{-1}$ (CHEN et al., 1985). Knowing the particle density, its aerodynamic diameter could be directly deduced as well as the particle concentration by unit volume. These data enabled calculation of mass and surface concentrations, the count median aerodynamic (M.M.A.D.), and the geometrical standard deviation (σg) of particle distribution.

All the preceding elements were made of brass or neoprene, and all angles, abrupt diameter changes and bifurcation were minimized in order to limit particle trapping.

<u>Subjects</u>

With the agreement of the Ethical Committee of the hospital Pitié-Salpêtrière 41 children from 5 to 15 years old, boys and girls, and 29 adults, men and women, volunteered the tests :

- all the children and a group of 10 adults (5 men and 5 women) were non-smokers and were healthy, as determined by both clinical examination and respiratory function explored by spirometry : vital capacity (VC), residual volume (RV), forced expired volume in 1 sec ; (FEV 1), total lung capacity (TLC), and functional residual capacity (FRC) ;

- a group of 9 adults (4 men and 5 women) suffered from interstitial lung disease (ILD) with restrictive lung function. A group of 10 adults (8 men and 2 women) suffered from chronic obstructive pulmonary disease (COPD) with obstructive lung function.

Methods

After calibration of flow-rates, volumes, generated aerosol sizes and concentrations, particle deposition was measured in the subject's airways.

1. Total airway deposition by mouth-breathing

Measurements were made during mouth-breathing, with a noseclip : first, with the mouthpiece closed, the spirometer was filled automatically with inhaled air, at a rate (V_T and fR) equal to that of previously determined controlled breathing. The A.P.S. then aspired air from the filled spirometer and measured the distribution and concentration (Ci) of particles to be inhaled. The subject then inhaled an identical air-particle mixture. After 5 washout cycles, the system was connected and the subject expired into the spirometer which was in turn emptied by the A.P.S., giving the distribution and concentration (Ce) of expired particles.

The deposition (Dm) of inert particles in the airways by mouth-breathing, was measured by comparing their concentration in the inhaled (Ci) and expired (Cem) aerosols :

$$Dm = 1 - Cem/Ci$$

2. Nasal deposition at inhalation

Following total airways deposition, some of the same healthy subjects, adults and children performed the tests during nose-breathing, through a nose-mask with the mouth shut; using the same procedure, and identical ventilation rate. The deposition (Dn) by nose breathing in the airways, was calculated in the same way as before, and two values were obtained :

- deposition by mouth-breathing : $Dm = 1 - Cem/Ci$
- deposition by nose breathing : $Dn = 1 - Cen/Ci$

(usually $Dn > Dm$).

It is usual to assume that the mouth has negligible air-flow resistance, and efficiency in removing airborne particles when breathing through a mouthpiece and wearing a nose-clip. So we may assume that Dm is equal to the sub-oral airway deposition, y, mainly the pharyngeal, tracheo-bronchial, and alveolar depositions, that stay the same during nose-

breathing, provided the subject breathe at the same flow-rate for the mouth and nose breathing, 1-Dn, is then equal to :

$$1-Dn = (1-x)\ (1-x')\,(1-y) \qquad \text{(Figure 2)}$$

In order to calculate the particle fraction deposited in the nose during inspiration, x, that indicates the efficiency of the nasal filter toward the various particles, we have to make the assumption that the two nose deposited fractions x and x' are equal, we have then :

$$1-Dn = (1-x)^2\,(1-y)$$
$$1-Dn = (1-x)^2\,(1-Dm)$$
$$Cen/Ci = (1-x)^2\ Cem/Ci$$

and finally, the "nasal particle filtering efficiency" is given by :

$$x = 1- \sqrt{Cen/Cem}$$

In these subjects, anterior rhinomanometric determination of nasal resistances as a function of nasal flow-rates was made for each side of the nasal passages (GAHEM and MARTINEAUD, 1985) : the pressure and flow-rates variations were measured simultaneously in both nares during inspiration. The flow-rates, $\mathring{Q}$, were measured with a n° 1 Fleisch pneumotachograph fitted to one nostril by a rubber piece, and connected to a membrane pressure-transducer. The pressure drop, p, over the opposite passage was measured in the vestibular region by a pressure recording catheter, connected to a second pressure transducer. The flow-rate and pressure signals were recorded in the x/y mode, for both nasal passages in turns. The right and left resistances Rr and Rl, could be calculated by the relationship :

$$R = p/\mathring{Q}$$

and the total resistance of the entire nose, Rr, by :

$$1/R_T = 1/R_r + 1/R_l$$

For a given total nasal flow-rate during inspiration, the partitioning of the air-flow between the two nasal passages was given by the maximal flow-rate values obtained during a quiet breathing in each side at the same moment.

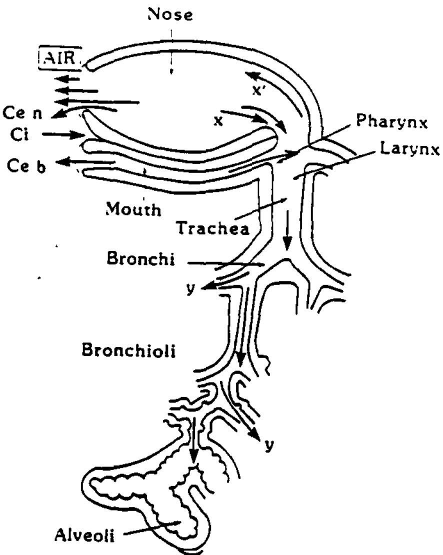


Fig. 2 : Regional deposition in the airways

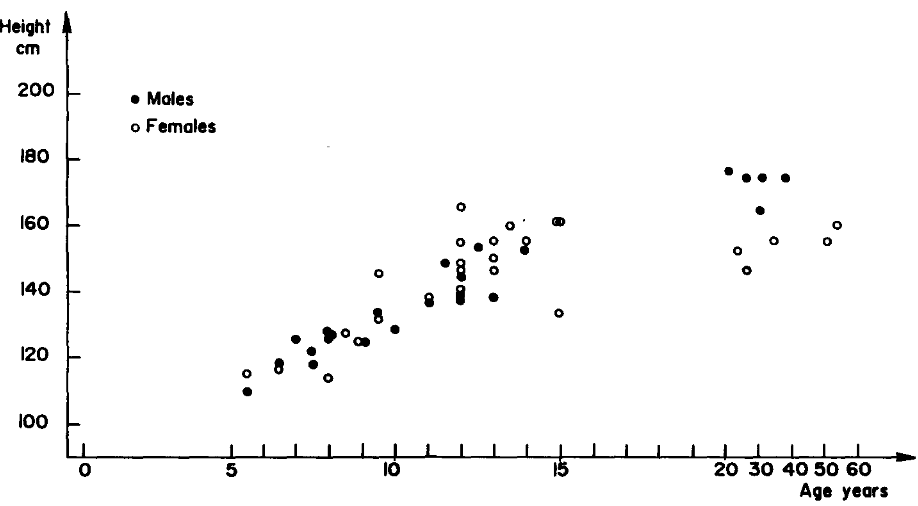


Fig. 3 : Body-size versus age in the healthy subjects

Table 2 Subjects' biometry and lung function

$\bar{M}$ · Mean σ · Standard deviation		11 children age ⩽ 8 y	10 children 8 < age < 12 y	20 children 12 ⩽ age ⩽ 15 y		10 healthy adults		9 ILD restrictive adults		10 COPD obstructive adults	
				M	F	M	F	M	F	M	F
Age (y)	$\bar{M}$	7.1	9.8	12.4	13.3	29.4	38.4	43.7	35.4	64.5	71.5
	σ	0.9	1.0	0.4	1.0	5.3	12.5	4.6	10.5	10.0	/
Height (cm)	$\bar{M}$	120.3	134.3	146.0	153.1	173.4	160.0	164.7	161.2	170.3	161
	σ	6.0	7.9	6.6	8.7	4.3	4.5	4.1	8.9	6.7	/
Weight (kg)	M	21.6	30.0	38.4	44.0	65.0	56.2	55.5	56.8	57.7	90.5
	σ	3.8	3.3	10.1	9.1	2.9	1.2	6.2	13.2	4.2	/
VC (l.)	$\bar{M}$	1.220	2.010	2.210	2.680	5.530	3.630	3.13	3.01	3.04	1.71
	σ	0.264	0.341	0.440	0.400	0.600	0.400	0.43	0.60	0.69	/
FEV_1 (l.)	$\bar{M}$	1.114	1.760	1.910	2.300	4.510	3.000	2.02	2.33	1.28	93.5
	σ	0.270	0.366	0.350	0.500	0.400	0.220	0.51	0.65	0.49	/
FRC (l.)	M	0.927	1.115	1.411	1.700	3.610	2.900	3.34	2.07	3.91	2.04
	σ	0.219	0.212	0.190	0.5	0.500	0.770	1.13	0.33	0.63	/

Table 3a Ventilation rates in the four subject groups under controlled resting ventilation

X : Mean σ : Standard deviation		11 children age ⩽ 8 y	10 children 8 < age < 12 y	20 children 12 ⩽ age ⩽ 15 y		10 healthy adults		9 ILD restrictive adults		10 COPD obstructive adults	
				M	F	M	F	M	F	M	F
Tidal volume (l.)	X	0.338	0.371	0.488	0.514	0.907	0.530	0.908	0.816	0.594	0.501
	σ	0.079	0.064	0.063	0.125	0.212	0.075	0.182	0.449	0.112	/
Inspiratory time (s.)	$\bar{X}$	1.07	1.26	1.33	1.47	2.21	1.80	1.31	1.06	0.99	2.25
	σ	0.36	0.35	0.44	0.32	0.60	0.26	0.48	0.44	0.31	/
Breathing cycle (s.)	$\bar{X}$	2.11	2.58	2.34	3.20	4.05	3.68	2.35	2.15	2.08	4.15
	σ	0.70	0.53	0.28	0.62	0.93	0.79	0.33	0.65	0.56	/
Mean inspiratory flow-rate ($l.s^{-1}$)	X	0.371	0.308	0.404	0.360	0.425	0.302	0.764	0.797	0.640	0.220
	σ	0.195	0.064	0.152	0.091	0.087	0.082	0.239	0.372	0.173	/

Table 3b Ventilation rates in the four subject groups under controlled moderate exercising ventilation

$\bar{X}$: Mean σ : Standard deviation		11 children age $\leqslant$ 8 y	10 children 8< age < 12 y	20 children 12 $\leqslant$ age $\leqslant$ 15 y		10 healthy adults		9 ILD restrictive adults		10 COPD obstructive adults	
				M	F	M	F	M	F	M	F
Tidal volume (l.)	$\bar{X}$	0.342	0.532	0.547	0.640	1.188	0.934	1.299	0.972	0.971	0.816
	σ	0.079	0.107	0.118	0.115	0.119	0.134	0.334	0.259	0.148	/
Inspiratory time (s.)	X	0.75	0.96	0.97	0.94	1.47	1.37	0.96	0.78	1.01	1.67
	σ	0.22	0.18	0.15	0.27	0.55	0.18	0.10	0.23	0.14	/
Breathing cycle (s.)	X	1.49	1.92	2.42	2.05	3.0	2.72	2.05	1.52	2.09	3.16
	σ	0.48	0.30	0.80	0.55	0.84	0.76	0.65	0.50	0.48	/
Mean inspiratory flow-rate ($l.s^{-1}$)	$\bar{X}$	0.484	0.572	0.595	0.724	0.894	0.697	1.355	1.404	0.978	0.509
	σ	0.145	0.147	0.246	0.218	0.223	0.150	0.328	0.631	0.202	/

Data treatment

The mean values and standard deviation of the biometric and respiratory function data are given on Table 2, for the subjects separated into six groups :

11 children age $\leq$ 8 years,
10 children 8 < age < 12 years,
20 children 12 $\leq$ age $\leq$ 15 years,
10 healthy adults,
9 ILD patients,
10 COPD patients.

The mean values and standard deviations of the ventilation parameters, at rest and at light exercise have been calculated for each of the 6 subject groups, tidal volume (l), inspiratory time and time of the total breathing cycle (s), and mean inspiratory flow-rate ($l.s^{-1}$). They are given on Table 3

For the healthy subjects, adults and children, the body size is plotted against age (Figure 3) and also given as histograms for age-groups (Figure 4) and so is FRC (Figure 5).

The deposition data have been treated differently for nose-breathing and mouth-breathing :

1. Nasal deposition during inspiration

Among the healthy subjects, 10 adults and 20 children performed the complete nasal and oral breathing : they were separated into three age-groups :

10 adults
8 children 12 $\leq$ age $\leq$ 15
12 children 5 < age < 12

The mean values and standard deviations of the ventilation parameters, at rest and at light exercise have been calculated for each of the subject groups :

- Q ($l.s^{-1}$) : mean inspiratory flow-rate ;
- R_T (kPa $l^{-1}s$) : total nasal resistance at this flow-rate ;

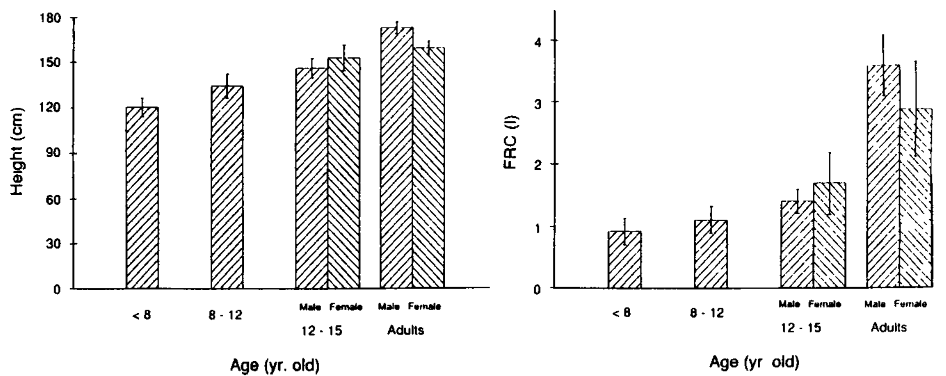


Fig. 4 : **Body-size** **Fig. 5** : **Functional Residual Capacity**

Biometric values of the healthy subject age-groups

Table 4

NASAL VENTILATION DATA IN THE THREE SUBJECT GROUPS DURING CONTROLLED BREATHING FOR NASAL INSPIRATORY PARTICLE DEPOSITION STUDY

$\bar{X}$ = Mean Value
σ = Standard deviation

RESTING VENTILATION

	10 adults		8 children age > 12 y		12 children age < 12 y	
	$\bar{X}$	σ	$\bar{X}$	σ	$\bar{X}$	σ
Mean inspiratory flow-rate ls^{-1}	.455	.222	.309	.084	.284	.130
Total resistances kPa $l^{-1}s$	.169	.06	.331	.151	.348	.262
Pressure drop kPa	.077		.102		.10	

MODERATE EXCERCISING VENTILATION

	$\bar{X}$	σ	$\bar{X}$	σ	$\bar{X}$	σ
Mean inspiratory flow rate ls^{-1}	1.057	.389	.522	.167	.466	.155
Total resistances kPa $l^{-1}s$	.269	.078	.471	.168	.484	.435
Pressure drop kPa	.284		.246		.225	

- p (kPa) : pressure gradient across the nose and nasopharynx during nose-breathing of the aerosol test (Table 4).

For the same inspiratory flow-rate, $\mathring{Q}$ choosen at 0.300 $l.s^{-1}$, each individual nasal resistances have been calculated, for the left and right nasal passages and for the total nose. All total resistances, as well as the ones of the maximal and minimal air-flow sides were plotted against age (Figure 6).

This presentation takes into account the nasal cycle with a predominant flow-rate in one of the nasal passages alternatively, that is observed in all of our subjects.

For all the individual values of each particle size deposited fraction, at rest and at exercise, linear correlation coefficients, r, were calculated with the corresponding flow-rates, $\mathring{Q}$, and total nose resistances, R_T (Table 5).

For every subject, and each deposition test, an impaction parameter, equal to :

$$\rho d^2 p^{2/3}, \text{ kg m}^{-1} \text{ Pa}^{2/3}$$

was calculated with :

ρ = inhaled particle density ($kg.l^{-1}$)
d = inhaled particle dynamic diameter (um)
p = pressure drop along the nasal passages (kPa)

These values were plotted against the individual values fo the nasal deposited fractions, for the three groups (Figure 7).

2. Total airway deposition by mouth-breathing

2.1. *For all the studied subjects*

A multivariate statistical analysis (PAGES et al., 1979) into principal components was computed upon all the individual subject's features :

- age, (y), height, (cm) ;
- ventilation represented by tidal volume V_T (l), inspiratory time, ti (s) ;
- respiratory function, VC, FRC, FEV1, (l) ;

Table 5

LINEAR CORRELATION COEFFICIENTS, r, BETWEEN NASAL PARTICLE DEPOSITION AT INSPIRATION

INSPIRATORY FLOW-RATES, $\dot{Q}$, AND TOTAL NASAL RESISTANCES, RT.

FOR 57 PAIRS OF OBSERVATIONS AMONG 30 SUBJECTS

MMAD µm	1	2.05	2.8
Inspiratory Flow-rates, $\dot{Q}$, $l.s^{-1}$	r = .357	.473	.435
Total Nasal Resistances, RT, $kPa l^{-1} s$	r = .066	-.148	- .254

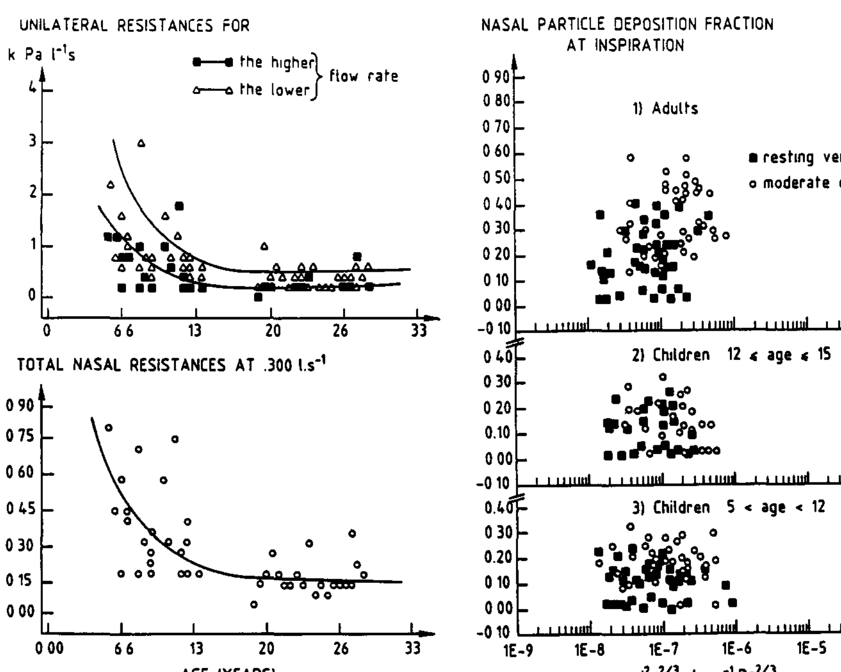


Fig. 6 : Healthy subject's nasal resistances versus age.

Fig. 7 : Individual nasal deposition versus the impaction parameter (rest + exercise)

- deposition of the three particle sizes, 1, 2.05 and 2.8 µm :
  - . RE1, RE2, RE3 at rest ;
  - . Ex1, Ex2, Ex3 at exercise.

This calculation was computed on IBM 3090-400, with the software APATDM from the mathematical library of the Compagnie Internationale de Service en Informatique(CISI).

This powerful method, resumed the 10 initial variables into 3 or more principal components, by which the 69 subjects were described with accuracy and simplicity. It provided linear correlations between those variables (Table 6), and a mutual comparison between the subjects : this was done on the graphs defined by the principal axes (Figures 8, 9) where were projected the subject points and the vectors of the variables.

Data for total deposition of each of the three adult groups healthy, restrictive, and obstructive were compared by the Mann and Whitney non parametric rank test (Figure 10).

2.2 *For the healthy subjects only*, adults and children, the breathing parameters actually measured during aerosol inhalation were plotted against age : tidal volume (Figure 11) and breathing frequency (Figure 12). Tidal volumes and respiratory times were also given as histograms for age-groups (Figures 13, 14).

The experimental deposition data were compared by C.P. YU to the calculated model he has developed (YU and DIU, 1983). This model of deposition continuous along the airways, integrates the three main deposition processess : impaction, sedimentation and diffusion of particles ; the calculation includes the particle characteristics :

- diameter, density, settling velocity, diffusion coefficient, etc... ;

as well as the morphological lung features :

- length and diameter of the different airway generations, and average inclination angle, according to Weibel's lung model A ;

and the breathing pattern :

- tidal volume, air velocity, respiratory pauses.

ACP : Representation of the subjects and variables on the graphs of the first principal axes for total deposition by mouth breathing.

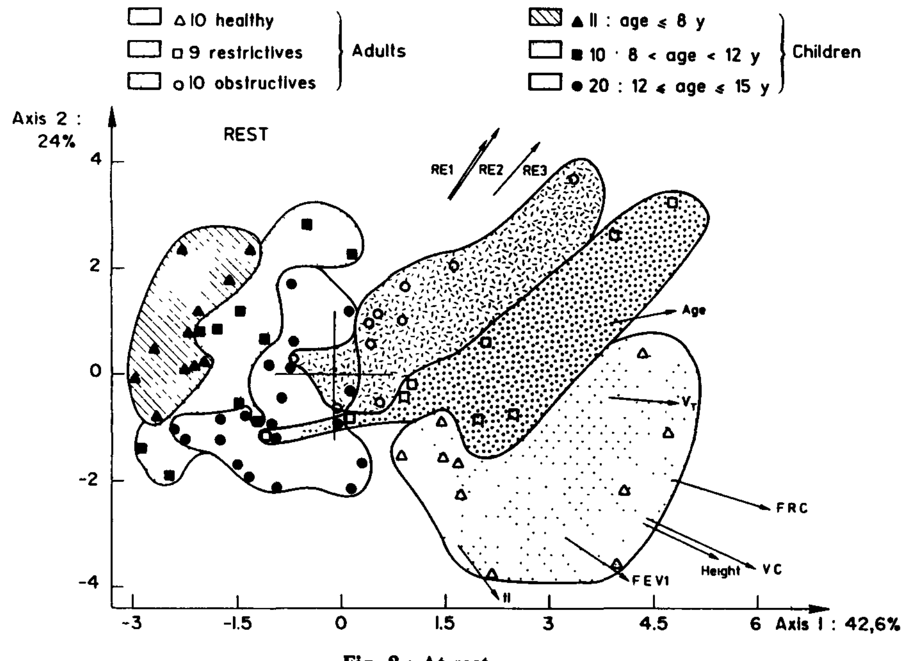


Fig. 8 : At rest.

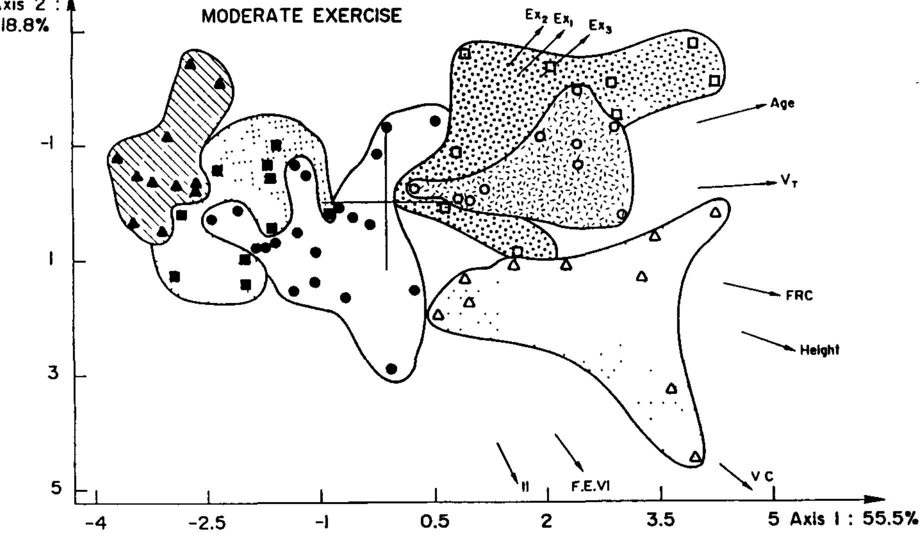
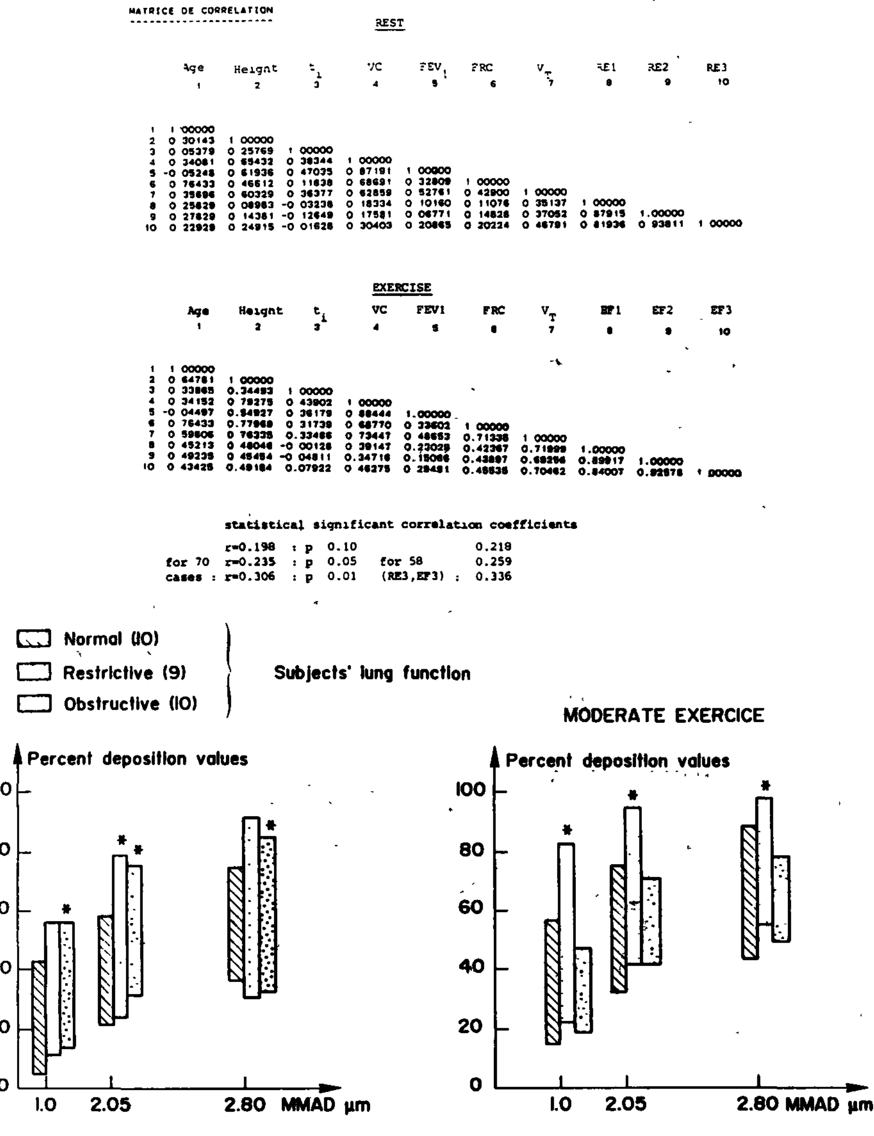


Fig. 9 : At moderate exercise.

Table 6 **ACP : Linear correlations between the 10 initial variables.**



MATRICE DE CORRELATION

REST

	Age 1	Height 2	t_i 3	VC 4	FEV_1 5	FRC 6	V_T 7	RE1 8	RE2 9	RE3 10
1	1 00000									
2	0 30143	1 00000								
3	0 05379	0 25769	1 00000							
4	0 34081	0 65432	0 38344	1 00000						
5	-0 05248	0 61936	0 47035	0 87191	1 00000					
6	0 76433	0 46612	0 11638	0 68691	0 32809	1 00000				
7	0 35696	0 60329	0 36377	0 62859	0 52761	0 42900	1 00000			
8	0 25629	0 08963	-0 03236	0 18334	0 10160	0 11076	0 35137	1 00000		
9	0 27629	0 14361	-0 12649	0 17581	0 06771	0 14826	0 37052	0 87915	1.00000	
10	0 22929	0 24915	-0 01626	0 30403	0 20665	0 20224	0 46791	0 81936	0 93811	1 00000

EXERCISE

	Age 1	Height 2	t_i 3	VC 4	FEV1 5	FRC 6	V_T 7	EF1 8	EF2 9	EF3 10
1	1 00000									
2	0 64781	1 00000								
3	0 33865	0.34493	1 00000							
4	0 34152	0 79275	0 43902	1 00000						
5	-0 04497	0.84927	0 36179	0 88444	1.00000					
6	0 76433	0.77969	0 31739	0 68770	0 33602	1 00000				
7	0 59606	0 76335	0.33486	0 73447	0 48653	0.71336	1 00000			
8	0 45213	0 48046	-0 00128	0 39147	0.23029	0.42367	0.71999	1.00000		
9	0 49235	0 45454	-0 04811	0.34716	0.15066	0.43897	0.69294	0.89917	1.00000	
10	0 43425	0.49184	0.07922	0 46275	0 29491	0.48536	0.70462	0.84007	0.92876	1 00000

statistical significant correlation coefficients

	r=0.198 : p 0.10			0.219
for 70	r=0.235 : p 0.05	for 58		0.259
cases :	r=0.306 : p 0.01	(RE3,EF3)	:	0.336

* : Patients' values significantly higher than the healthy subject's p ≤ 0.05 by the Mann and Whitney non-parametric test

Fig. 10 : Total deposition by mouth-breathing ; ranges for the healthy and pathological adults.

Experimental ventilation values in healthy subjects for mouth-breathing.

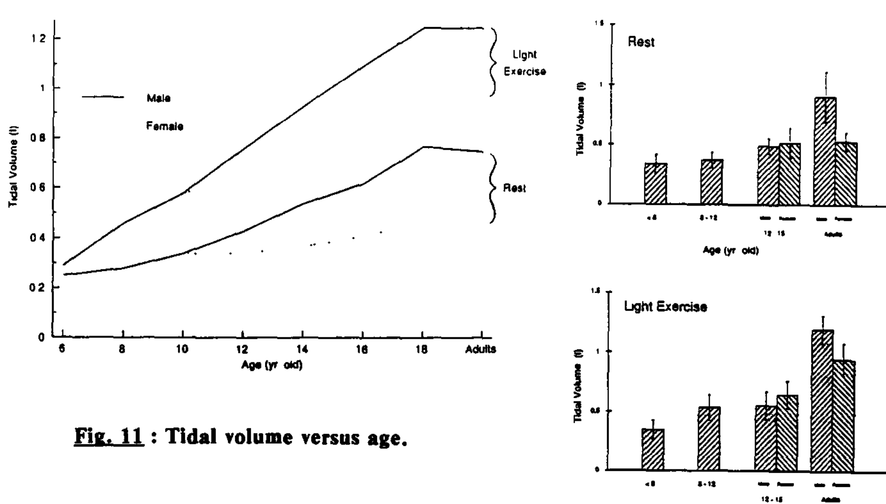


Fig. 11 : **Tidal volume versus age.**

Fig. 13 : **Tidal volume in age-groups.**

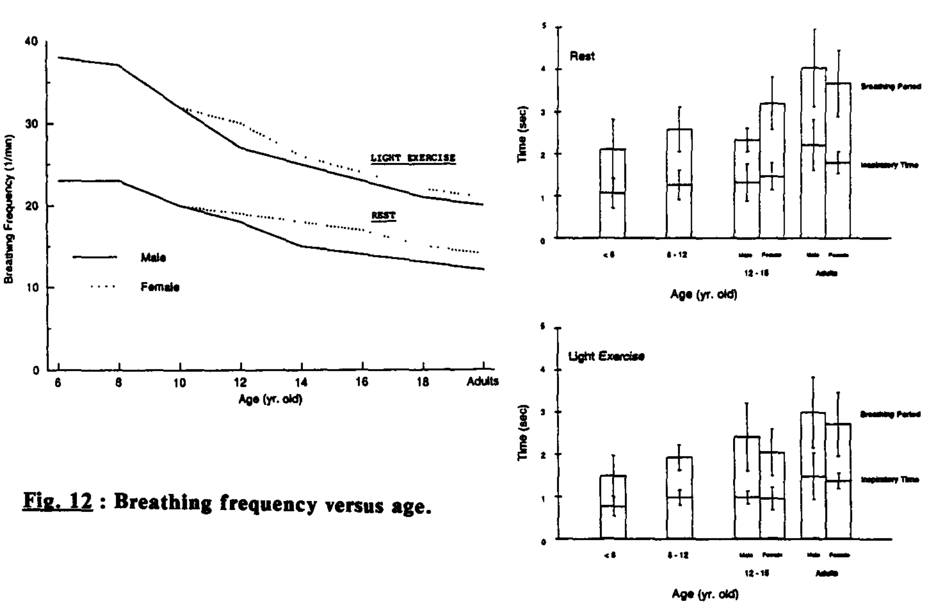


Fig. 12 : **Breathing frequency versus age.**

Fig. 14 : **Breathing cycle in age-groups.**

An age-dependant model transformation (XU and YU, 1986) was later on based upon the growth processes in lung geometry described quantatively by REID (1977), from birth to adulthood. This growing pattern was applied to the thoracic airways :

- tracheobronchial tree, fully developed at birth in number and growing only in sizes, and for which the ratio of length to diameter is constant except for the trachea and the main bronchi ;

- alveolar region, where the total number and size of alveoli is strongly dependent on age.

For the comparison with our experimental data, Professor YU, integrated the growing pattern in body size versus age of our children subjects ; he calculated deposition at rest and under exercise, using the exact breathing parameters recorded during the experiments.

He kindly provided several figures illustrating this comparison :

- the experimental data of deposition are presented for each age-group as histograms, framing the calculated values, at rest and at exercise (Figure 15) ;

- he plotted all the calculated fractions against the experimental data for the children separated into two-year age intervals : 5 to 7, 7.5 to 9.5, 10 to 12 and 12.5 to 15, (Figures 16 to 19) and for adults (Figure 20) ; on each graph is presented the regression equation obtained ;

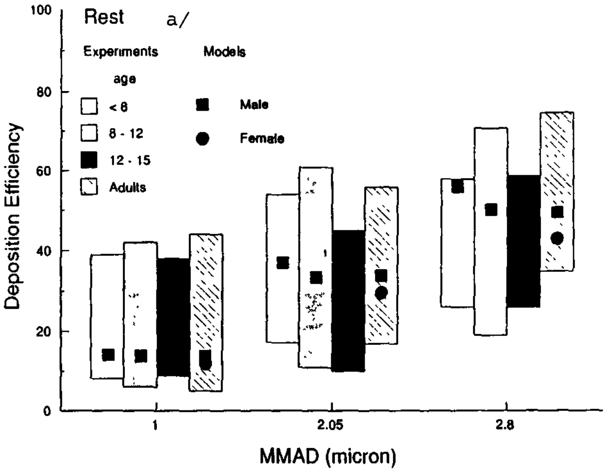
- finally, the ratio of model over experimental deposition was plotted against age for each particle size (Figures 21 to 24), both at rest and at exercise.

The ratios of deposited fractions of each particle size over the corresponding inhaled tidal volume of aerosol were calculated for each subject. This deposition by unit inspired volume was plotted against body size at rest and at exercise (Figures 25, 26, 27).

RESULTS

1. Nasal deposition at inspiration : nasal filtering efficiency

In children the nasal resistances at a flow-rate $\mathring{Q} = 0.300\ l.s^{-1}$ were decreasing sharply with increasing age (Figure 6).



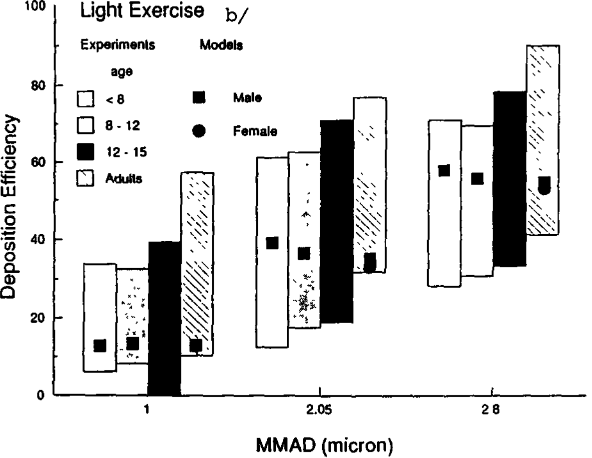


Fig. 15 : Total deposited fractions by mouth-breathing : model predictions and ranges of experimental data.

a) At rest

b) At moderate exercise

Regressions of experimental data against model predictions in total deposition by mouth-breathing (rest + exercise).

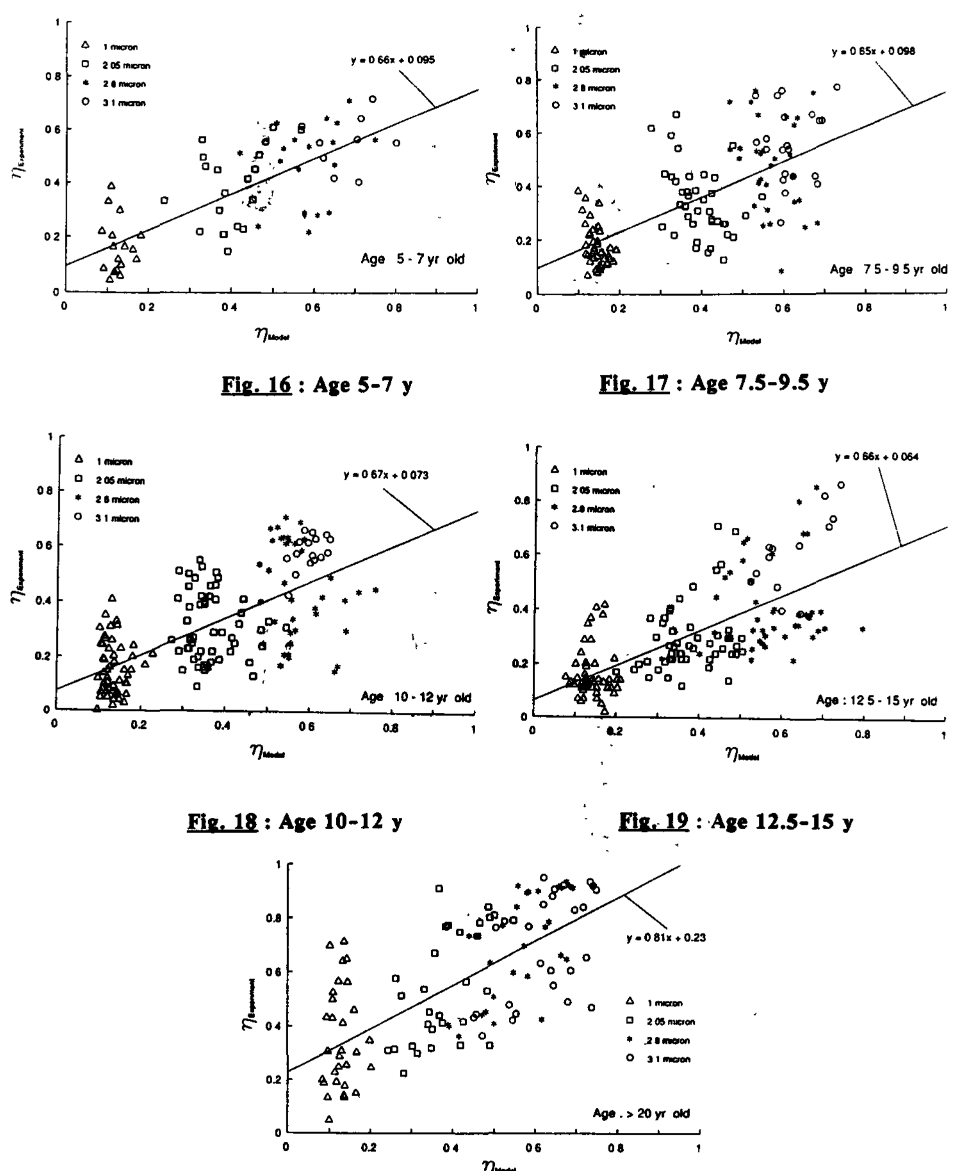


Fig. 16 : Age 5-7 y

Fig. 17 : Age 7.5-9.5 y

Fig. 18 : Age 10-12 y

Fig. 19 : Age 12.5-15 y

Fig. 20 : Adults

Ratio of predicted to experimental total deposition by mouth-breathing (rest + exercise).

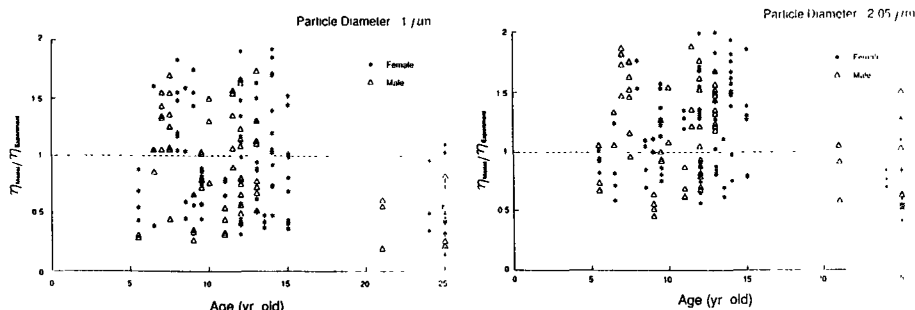


Fig. 21 : 1 um

Fig. 22 : 2.05 um

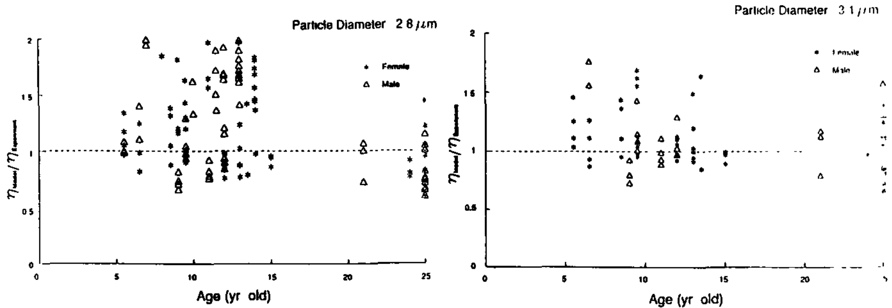


Fig. 23 : 2.8 um

Fig. 24 : 3.1 um

Deposited fractions by unit tidal volume during mouth-breathing versus body-size : a) At rest ; b) At moderate exercise.

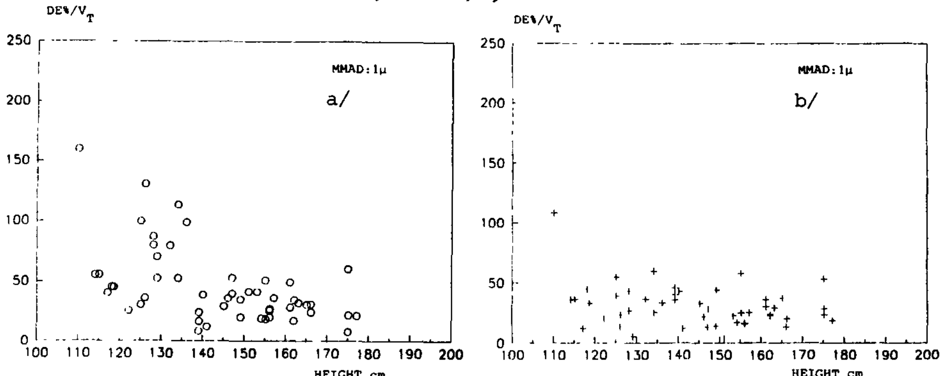


Fig. 25 : 1 um

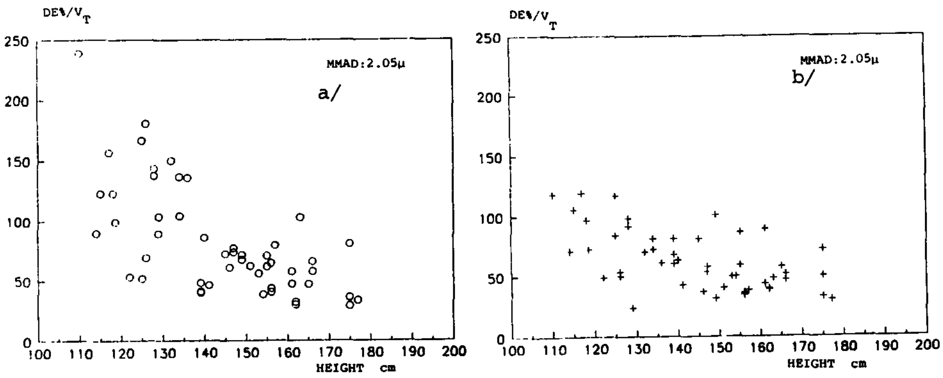


Fig. 26 : 2.05 um

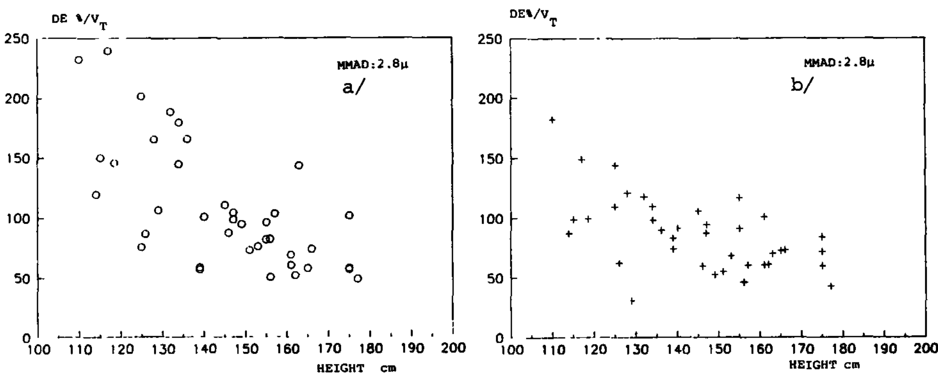


Fig. 27 : 2.8 um

The adult values were around 0.150 kPa $l^{-1}s$ for total resistances, and were scarcely reached at 14 years. The nasal cycle, with a predominant flow-rate in one of the nasal passages alternatively, existed in all of our subjects, and compared to the adults, the youngest children had a much more important desequilibrium between the two unilateral resistances.

For each of the three age groups, the deposition data plotted against the impaction parameter $\rho\ d^2 p^{2/3}$ (Figure 7) were very dispersed. However, the variability of nasal percent deposition among adults is greater than that in the two child groups in most cases, which suggests that the children were probably as successful in reproducing flow during oral and nasal breathing, a necessary condition to accurately calculate nasal percent deposition. For the studied particle sizes, the children in both groups had obviously less nasal particle filtering efficiency than the adults. Because the pressure drop, p, along the nasal passages, is dependent on the nasal resistance, R_T, and on the inspiratory flow rate, $\mathring{Q}$, it was logical to try to delineate the respective influences of these two parameters upon deposition. For the data as a whole, adults and children, at rest and at exercise, the linear correlation of each particle size deposited fractions was higher with the flow-rates (as an example, for 2.05 µm, MMAD, r = 0.48) than whith the total nasal resistance (r = 0.15), (Table 5). From Table 4 : one can notice that the adults increased, more their flow-rates from resting to exercising ventilations (57 %) than did the children in both groups (41 and 39 %) ; this contributed a higher progress in pressure (73 % for the adults, 59 and 56 % for the children).

2. Total airway deposition by mouth-breathing

On the graphic representation of the multivariate analysis (ACP) (Figures 8 and 9) was indicated the percentage of the total information resumed by each of the two first principal axes (46.6 and 24 % at rest, and 55.5 and 18.8 % at exercise). The relative position on the graphs of the groups of subjects indicated their mutual proximity regarding the listed variables. The general disposition of the subjects and variables was the same at rest and at exercise : Axis 1 was positevely correlated with all the variables, and Axis 2 separated the deposition variables from the functional ones. The age and tidal volume were almost confounded with Axis 1, the best resuming factor, and therefore were excellent descriptive variables of our subjects. However the body size, better correlated with the lung function than age, was a good index for morphology and lung function : it could be a valuable substitute for age in a possible relationship with deposition. The correlation values (Table 6) confirmed the graph observations : as an example, height was better correlated with VC (r = 0.65), than was age (r = 0.34) ; at rest V_T was significantly correlated with all the 9 other variables (r = 0.35 to 0.63), the correlations values were even higher for exercise

where deposition data presented high positive correlation coefficients with V_T (0.69 to 0.72) and good positive values with age (0.43 to 0.49), with height (0.45 to 0.49) and lung function : VC (0.35 to 0.46) and FRC (0.42 to 0.45) ; they were faintly correlated with FEV1, and not at all with the inspiratory time.

On the graphs, the subject group were well individualized : the children were classified, regarding Axis 1, according to their age, and all the adults stayed at the same level. Along Axis 2, the younger children, like the restrictive and obstructive patients were higher than the healthy adults, especially at exercise, indicating that they increased their deposition independently from their lung function.

In pathological subjects

The non-parametric rank test of MANN and WHITNEY upon the individual deposited fractions in the three adult groups (Figure 10) showed that, at rest, the obstructive patients deposited more than the others, and that, at exercise, it was the restrictive ones, but the significance is not high ($p < 0.05$) probably because of the small numbers of subjects. This result, was observable on the ACP graphs, where the relative positions of the obstructive and restrictive groups have changed from rest to exercise.

In the healthy subjects the minute ventilations at rest and at exercise, resulted of changes in breathing rates : in the younger children, ventilation was increased at exercise more by frequency (Figures 12 and 14) than by tidal volume (Figures 11 and 13) ; the adult like increasing pattern was reached around 15 years. Figure 15 shows that the observed deposition values were not very different in adults and children at rest, and rather higher in adults at exercise ; this could result from that higher increase in V_T.

The experimental values were not constantly lower or higher thant the model ones. They seemed to agree better for particles of 2.05 and 2.8 µm than for 1 um, depending on the age group. The regressions for experiment in function of model was better for adults (slope = 0.81) on Figure 20 than for either of the children groupes (slope ≈ 0.66) on Figures 16 to 18. Plotted against age, the children experimental data of 1 µm fitted better the model than the adult's (Figure 21), but this changed progressively with increasing particle sizes, and the fitting was better for adults with 2.8 and 3.1 µm (Figure 23, 24). As it was observed on the ACP, and from the correlation coefficients, there was a strong dependence of deposition upon tidal volume ; this might have outweighed an eventual effect of age and growth of the lung. Indeed such effect was suggested by Figures 25, 26 and 27 where deposition by unit of inspired aerosol volume was plotted against body size,

the best index of lung growth in our calculations : this ratio decreased with increasing body size, and its values seemed higher at rest than at exercise, especially for 1 µm.

DISCUSSION

The conducting airways may be considered as a protective system which prevents large particles from penetrating into the alveolar region. This "filtration" is determined by the three mechanisms : diffusion, sedimentation and impaction. It depends on the size of the particles, on the airway dimensions and on the breathing pattern.

Because of smaller airway dimensions, it is likely that the relative effects of impaction and diffusion for a given particle size are more effective in the child but that sedimentation onto the surface of the mucous layer due to gravity has about the same effect as in the adult.

Several calculated models predicted a greater extra-thoracic and tracheobronchial efficiency in smaller (younger) than in larger (older) airways, assuming that conductive airways of the child therefore form a more efficient protective mechanism than in the adult, since they are so much smaller and should trap particles much more easily. However the effect of the change in lung size on the "filtration" of aerosols is surprisingly small. The child airways are certainly narrower but they are also shorter, and the time available for sedimentation or diffusion is reduced by the higher rate of breathing. It appears, therefore that the alveoli of the child are protected to about the same degree as those in the adult.

1. Nasal deposition at inspiration

In the nose, deposition by impaction is predominant : in attempting to correlate nasal deposition to an impaction parameters, YU et al. (1981) used the factor $\rho \, d^2\mathring{Q}$, where :

ρ = particle density
d = particle diameter
$\mathring{Q}$ = inspiratory flow-rate

The significant scatter when using such a parameter was noted by YU, as it was by HOUNAM et al. (1971) who obtained a better correlation with $\rho \, d^2 p$, (with p = pressure drop in the nose) probably because p is a function of both flow-rate and a dimension.

HEYDER and RUDOLF (1977) found that data from their adult studies could better correlate by the parameter $\rho\ d^2p^{2/3}$, which was tested in the present study for adults and children.

It was proposed by PHALEN et al. (1990) that pressure drop as a correlating factor for nasal deposition could be extended to children. This hypothesis was accompanied by experimental studies in scaled, idealized physical models of the nasal passage, following the design of SCOTT et al. (1978), of an adult and a newborn child. These studies demonstrated that deposition percent (for a range of flow rates) when correlated with $\rho\ d^2\dot{Q}$ showed different curves for adult and child, but when correlated with pressure drop, a single curve for adult and child was obtained. It should be noted that a single idealized model was employed for each adult and newborn child, based on the date of SCOTT et al. (1978). The flow resistance of the idealized adult and child models were similar to values measured in vivo.

The observations of the present study, in which nasal deposition was measured in adults and children in vivo, are not in agreement with the conclusions of PHALEN et al. (1990) in that deposition gives a much higher correlation in adults and children with flow rate than with nasal pressure drop. Neither parameter gives a high correlation coefficient, indicating that nasal passages shape differences within and between age groups are such as to make it difficult to predict nasal deposition accurately solely based on flow rate or resistance.

This study indicates that the age effect of nasal deposition for normal breathing is not a monotonic function of age from 5 to adulthood : children of teenage show lower nasal deposition for two particle sizes. At moderate exercise flowrate, both teenage and younger children have lower nasal percent deposition of 1 micrometer particles on average than adults. For the other size, only the teenage children showed significantly lower percent deposition. It would be useful to have a more detailed measurement of nasal passage dimensions in future studies, such as the method of acoustic rhinometry described by HILLBERG et al. (1989). This technique measures the cross-section area of each nasal passage which plays an important role in inertial deposition, because it is related to the air velocity at each point in the nasal passage, and the region of highest deposition probability is that where maximum velocity undergoes a geometric situation optimal for deviation of particle paths from flow streamlines. Deposition in the anterior nasal passage, near the site of minimum cross-sectional area is consistent with the studies in nose models reported by ITOH et al. (1985).

2. Total airway deposition by mouth-breathing

Our "total deposition" refers to tracheobronchial and alveolar ones, excluding nasal deposition. For these particle sizes, deposition occurs in the tracheobronchial tree by impaction and sedimentation, and in alveoli mostly by sedimentation. Undee these conditions, the evaluations of total particle deposition in adult airways from inhalation experiments, have pointed out its relationship with tidal volume and inspiratory time (HEYDER et al. 1980). If our data of deposition were strongly dependent on tidal volume, the inspiratory time, at least in the range of our experiments, did not influence them ; this could be explained by the ordinary cyclical pattern of ventilation, with peak-flows and very small inspiratory pauses, of our subjects, in contrast to the constant "academic" air-flow adapted by HEYDER et al.. According to YU and DIU (1983) small values of ti, like in our study could not subtantially influence deposition in cyclic breathing pattern.

Authors have pointed out the large intersubject variability of total airway deposition (HEYDER et al., 1982) ; the good correlations obtained in this study between deposition data and lung volumes, VC and FRC, as well as with age and body size, could at least explain part of this variability ; this is obvious for restrictive patients, who deposited more than the healtly subjects with similar tidal volumes.

In the obstructive subjects, despite smaller tidal volumes, deposition was higher than in healthy subjects, at least for ventilation at rest. A similar observation was made by KIM et al. (1988) using an aerosol of 1 µm particles. He related the data to the narrowing of conductive airways resulting in increased impaction "in situ" together with irregularities in flow-distribution and lung penetration of aerosol, increasing peripheral deposition in several zones, therefore enhancing overall aerosol lung deposition.

In the healtly subjects, the fitting of our data to YU's model was better for adults than for children as shown on Figures 16 to 20. In children the fitting was better for the smaller particles (1 and 2.05 µm), than for the larger ones (2.8 and 3.1 µm) for which the experimental data are higher than the model ones this might result from a greater impaction under cyclic breathing in an experiments, than under the constant air-flow postulated in the model.

In any event, the age factor child/adult effect upon deposition was not predicted to be large for these particle sizes (1.2 at 5 years old and 1.1 at 10 years, XU and YU, 1986) and this was behind our data dispersion. This is probably why the fittings of experimental data to the model were acceptable but not excellent. Particle deposition studied in adults

and children under physiological conditions close to spontaneous ventilation were better correlated with ventilation (V_T) than with lung dimensions (VC and FRC), probably because the latter included anatomical parameters that were not measured by lung function testing. However, when normalized by unit of tidal volume the deposition data obviously decrease with increasing body size ; this indicate that a meaningful relationship between aerosol deposition and lung dimensions, would have been possible only if the breathing pattern has been kept constant for all ages.

CONCLUSION

This study has dealt, in normal conditions of ventilation, with deposition of particles from 1 to 3 um inhaled by healthy adults, patients with impaired lung function and children from 5 to 15 years of age. Compared with healthy adults, patients deposited more. In children, deposition did not differ much from that in the adults : children have airways which are structurally mature and therefore just miniaturized ones of an adult. The lower ventilation, on the one hand, prevents more particles from entering the airways, but on the other hand, the smaller lung dimensions would increase deposition probabilities. However, this is probably a much too simplified representation of the effect of growth upon deposition. In order to precise the important role of lung dimensions, former studies should keep ventilation constant for all ages and body sizes, and include morphometric measurements at least of the nasal passages that are the easiest to reach.

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   D-6000 FRANKFURT/MAIN (R.F.A.)

V. Publications :

M.H. BECQUEMIN, M. ROY, S. HERSON, P. GODEAU, A. TEILLAC. Clairance tracheobronchique à court-terme dans le syndrome de Gougerot-Sjögren apparemment pur. Bull. Eur. Physiopathol. Respir. 1986, 22, 551-557.

M.H. BECQUEMIN, M. ROY, D. ROBEAU, S. BONNEFOUS, J. PIECHOWSKI, A. TEILLAC. Inhaled particle deposition and clearance from the normal respiratory tract. Respir. Physiol. 1987, 67, 147-158.

A.C. JAMES, M. ROY. Dosimetric lung models in "Age-related factors in radionuclide metabolism and dosimetry". G.B. GERBER, H. METIVIER, H. SMITH Ed. 1987 : 95-108. Martinus Nijhoff, DORDRECHT for C.E.C.

M. ROY, M.H. BECQUEMIN, S. BONNEFOUS, A. BOUCHIKHI, E. PATTY, A. LAFUMA, J.C. PERRIER. Clinical study of inhaled aerosol total deposition in healthy adults and children in "Age-related factors in radionuclide metabolism and dosimetry". G.B. GERBER, H. METIVIER, H. SMITH Ed. 1987, p. 79-86. Martinus Nijhoff, DORDRECHT for C.E.C.

A. BOUCHIKHI, M.H. BECQUEMIN, J. BIGNON, M. ROY, A. TEILLAC. Particle size study of nine metered dose inhalers, and their deposition probabilities in the airways. Eur. Resp. J. 1988 - 1, 547-552.

M. ROY, D.L. SWIFT, M.H. BECQUEMIN, A. GAHEM, A. BOUCHIKHI, D. RODRIGUE. Nasal resistances and particle nose deposition in healthy adults and children. Journal of Aerosol Medicine, 1988 Vol. 1, n° 3, p. 236.

M.H. BECQUEMIN, A. BOUCHIKHI, M. ROY, J. CARLES, A. TEILLAC. Total particle deposition in the normal respiratory tract : influence of ventilatory parameters. Journal of Aerosol Medicine, Vol. 1, n° 3, 1988, p. 191.

A. BOUCHIKHI, M.H. BECQUEMIN, C. HARPEY, M. ROY, A. TEILLAC. Fusafungine : Aerosol droplet features and total deposition in obstructive and healthy subjects' airways. Journal of Aerosol Medicine, Vol. 1, n° 3, 1988, p. 246.

M.H. BECQUEMIN, B. DAUTZENBERG, A. BOUCHIKHI, M. ROY, F. ANTOUN, A. TEILLAC. Optimization of Pentamidine aerosol administration for acute lung disease treatment. Journal of Aerosol Medicine, Vol. 1, n° 3, 1988, p. 244.

M. ROY. Lung clearance modeling on the basis of physiological and biological parameters. Health Physics, vol. 57 sup. 1, 1989, in press.

RADIATION PROTECTION PROGRAMME

Final Report

Contractor: Contract no.: BI6-D-201-F

Université Paul Sabatier
Faculté de Médecine Purpan
Allées Jules Guesde, 37
F-31073 Toulouse Cédex

Head(s) of research team(s) [name(s) and address(es)]:

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F-31073 Toulouse Cédex

Telephone number: 061-25.21.23

Title of the research contract:

Biochemical and biophysical studies on the effects of very low doses of ionizing radiation on cells.

List of projects:

1. Biochemical and biophysical studies on the effects of very low doses of ionizing radiation on cells.

Title of the project no.: B16 - D201F

Biochemical and biophysical studies on the effects of very low doses of radiation on cells.

Head(s) of project:

Pr. H. PLANEL* - Laboratoire de Radiobiologie et Biologie spatiale - Faculté de Médecine - TOULOUSE -

Scientific staff:

F. CROUTE, Y. GAUBIN and B. PIANEZZI (Toulouse University)

I. Objectives of the project:

Contrary to high doses of ionizing radiation, effects of very low doses, less than 0.1 Gy, are not well known. However, previous experiments have shown that very low dose rates of chronic irradiation can stimulate the proliferative activity of Paramecia and Cyanobacteria (PLANEL et al. Health Physics, 1987). We intend to investigate, in this programme, the effects of very low doses of gamma rays on human cells cultivated in vitro.

II. Objectives for the reporting period:

In view to estimate the possible effects of the maximum radiation dose to which people could be safely exposed (0.05 Gy/year), we intend to investigate the effects of a dose rate of 0.05 Gy/week on carbohydrate metabolism of human dermal fibroblasts. The rate of glucose utilisation will be investigated through the enzyme activities of pathways of the pentose phosphate shunt (glucose 6 P dehydrogenase) and of the glycolysis (glyceraldehyde 3 P phosphate dehydrogenase and pyruvate kinase). In another way, the transmembrane resting potential (TMRP) will be measured.

III. - Progress achieved :

I. - METHODOLOGY

Cell cultures : human diploid dermal fibroblasts were cultivated as a monolayer in 10 % calf serum-supplemented MEM.

Irradiation facilities : cultures were exposed to a 60^{Co} source giving a dose rate of 6.25 mGy/day at the culture level.

Biochemical investigations: After appropriate times of irradiation, cells were trypsinized, washed twice in PBS and disrupted by 30s of sonication. The homogenats were centrifuged to clarity at 5 000 g (0°C, 5 min.) and the supernatants used for assays.
Protein content was determined acccording to Lowry's method (1951). The glucose 6 phosphate dehydrogenase (G6PDH) was assayed according to the method of Lohr and Waller (1974). Glyceraldehyde 3 phosphate dehydrogenase (G3PDH) was measured using the method described by Scoper R.K. (1969) and the assay of pyruvate kinase was carried out as described by Gutmann and Bernt (1974).

TMRP measurements were performed with glass microelectrodes under a microscope. About 2000 single cell measurements were done for each culture condition.

2. - RESULTS

The results of 4 experiments are exposed in table 1.
Irradiation did not induce significant change in G6PDH activity throughout the cell cultures. However, the G3PDH and pyruvate kinase activities appeared inhibited during the exponential growth phase (4th day of cultures) differences are significant ; $p < 0.05$. Enzyme activities were recovered at the 7th day. In another way, cells did not show changes of their TMPR values under irradiation as compared to controls.

3. - DISCUSSION

Our investigations show that, in our experimental conditions, gamma rays decrease the activity of enzymes involved in the glycolysis • pathway.
The G3PDH is a key enzyme catalyzing the single oxidative step in glycolysis. The oxidation, under irradiation, of the thiol group essential for its catalytic properties, could lead to the observed slight inactivation of this SH-enzyme. This hypothesis is in good agreement with results of Palecz and Leyko (1983) showing that ionizing radiation doses of 5 Gy cause a slight decrease in SH groups of human erythrocyte membrane that may be responsible for the inactivation of membrane bound SH-enzymes. Bartoc et al. (1975) found a decrease of activities of several other enzymes involved in glycolysis (hexokinase and phosphofructokinase) which is linked to the oxidation of their SH group. We can suppose that the decrease of pyruvate kinase activity could be ascribed to a general decrease of the glycolysis pathway activity. It should be observed that there is a recovery of all enzyme activities at the end of culture.
Short-term control of the pentose phosphate cycle is determined by G6PDH activity which depends on GSH/GSSG ratio. We can assume that low

irradiation doses used do not cause significant change of the glutathione status in the cells as early expected. Indeed, we found no change of catalase and glutathione reductase activities under irradiation (cf our last report). The lack of TMPR change is in favour of an undisturbed membrane permeability.

	4th day (25 mGy)		7th day (54 mGy)	
	CONTROL	IRRADIATED	CONTROL	IRRADIATED
G6PDH (U.I)	52.2 ± 1.2	53.2 ± 3.1	80.5 ± 3.4	78.8 ± 7.6
G3PDH (U.I)	219.0 ± 18.2	178.1 ± 21.1	412.5 ± 23.4	415.2 ± 37.7
Pyr. Kinase	366.5 ± 16.1	282.6 ± 38.1	702.4 ± 16.0	727.7 ± 20.9
TMRP (eV)	10.5 ± 1.8	9.9 ± 2.3	10.2 ± 2	9.4 ± 4.9

Table 1

In CONCLUSION

Very low doses (about 25 mGy) can bring changes in the metabolism of human cells. However, these responses are transient and disappear even when cultures are yet irradiated. On the other hand, it can be pointed out that the very low doses induce an enzyme inhibition : the radiation hormesis, already reported in single-cell organisms, did not occur under our experimental conditions.

Title of the project no.: B16 - D201F

Biochemical and biophysical studies on the effects of very low doses of radiation on cells.

Head(s) of project:

Pr. F. STEINHAUSLER - Institüt für Biologie, biochemie un biophysik - SALZBURG -

Scientific staff:

B. RUBEL, M. MEISS, C. ATZMULLER and M. HUBER (Salzburg University)

I. Objectives of the project:

INTRODUCTION

The validity of different models for extrapolation of dose effect curves from high-level to low-level dose areas is questionable. The aim of the present investigation is therefore to give more detailed information about the reaction of cells in the low dose region using in vitro cultures.

The parameter of investigation chosen was the protein pattern of the cytosol and the membrane, which is dependent on the gene expression and thus can be changed by the influence of an external parameter like chronic low level ionizing radiation.

II. Objectives for the reporting period:

In this part of the project SDS-gel-electrophorese measurements of the cytosol and membrane protein patterns of human fibroblasts in culture at different ages and levels of development, irradiated with dose rates of 72 μGy/day and doses of 3.4 mGy, 6.6 mGy and 14 mGy, were carried out and compared to control measurements.

III. Progress achieved:

I. - METHODS

Material

Human skin fibroblasts were cultivated as a monolayer in flasks. From the primary cell strain subcultures were prepared containing a heterogenous cell population with cells in different phases of the cell cycle.

Cultivation method

Skin fibroblasts were cultivated with Eagle's BME with Hepes Buffer (20 mM) + 10 % foetal bovine serum, 1 % non-essential amino acids, 1 % glutamine, 1 % penicillin + streptomycin, at 37 degree C in an incubator.

Irradiation facilities

The cell cultures were exposed for 47, 92 and 195 days to a planar source containing thorium-nitrate by growing the cells in culture flasks 10 cm above the irradiation source (dose rate : 72 µGy/day). Unirradiated control cultures were kept under the same cultivation conditions.

SDS-Polyacrylamid-Gel-Electrophorese Measurements

In order to measure the protein patterns of the cellular cytosol and membrane fraction after irradiation, the cells in the culture flasks were suspended and prepared for the electrophoresis procedure. The same protocol was carried out with control cells.

Evaluation

To evaluate the molecular weight of the protein bands, a calibration standard was run in parallel with different samples.

II. - RESULTS

Three experiments have been performed. In two experiments, no changes have been noted. However, in a third one, using the same technics, changes in membrane proteins were observed, as shown in the following report. Three series have been carried out with about 8 x 10^7 skin cells each time. One control series was established at the beginning. The cells of the second series for 92 days (total dose : 6.6 mGy) and of the third series for 195 days (total dose : 14 mGy).
The protein pattern of the cytoplasm showed no change over the whole observation period and was comparable to the control cytoplasm (fig. 1). The protein pattern of the membrane fraction was changed in the first and the second series ; the protein concentration of the third series was too low for detection. Comparison with the unirradiated control proteins showed that in the first series (3.4 mGy) two membrane proteins with the molecular weight of 45 and 50 kD are produced in higher amounts in contrast to a band of 24 kD, which was not detectable in this lane. In the second series (6.6 mGy), a band of 60 kD appeared together with a double-band instead of a single one at 45 kD. After irradiation with 6.6 mGy, the 24 kD protein again can be demonstrated at a normal level. Additionally, the expression of a molecular weight range between 12 and 18 kD increased in this group (fig. 2).

Figure 1: Protein pattern of the cytosol of human skin fibroblast cells

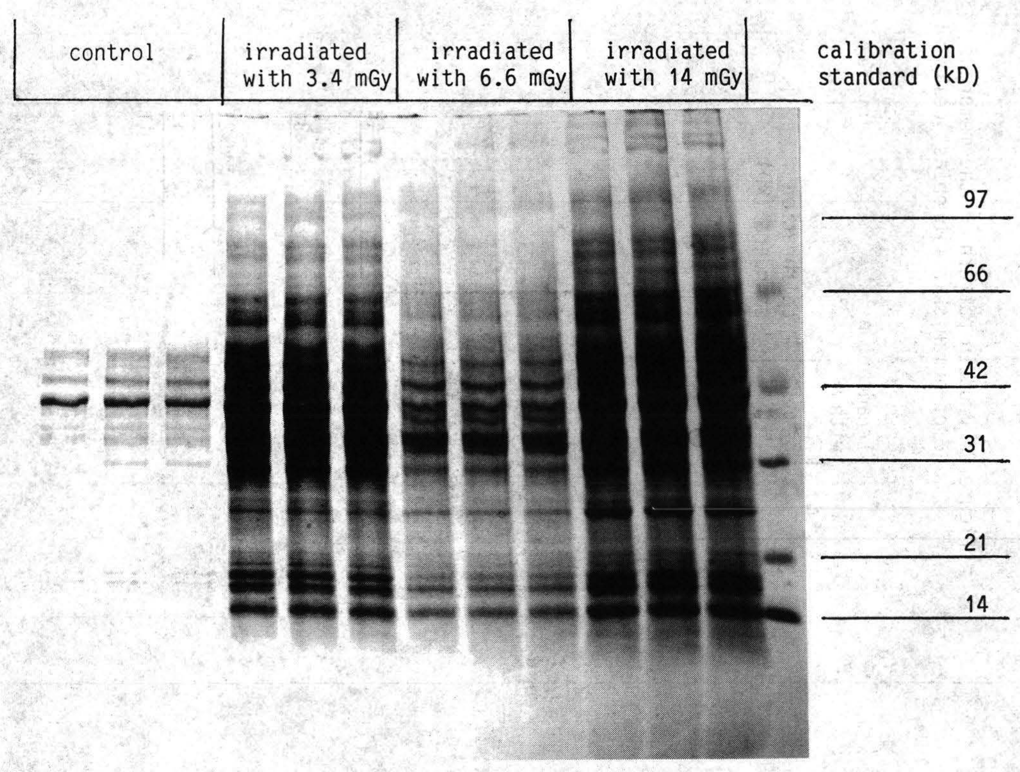
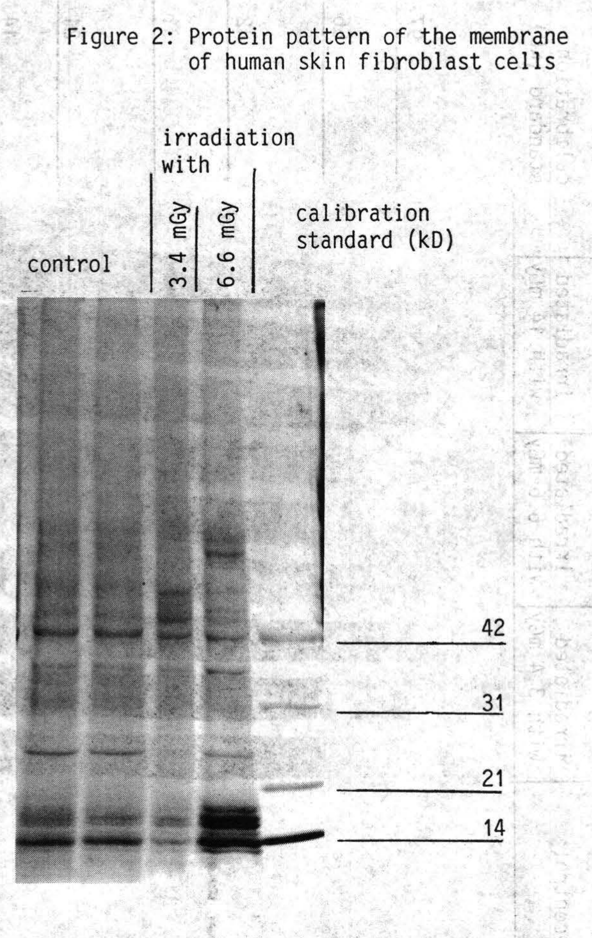


Figure 2: Protein pattern of the membrane of human skin fibroblast cells



RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-102-UK

United Kingdom Atomic Energy
Authority
Atomic Energy Establishment
Winfrith, Dorchester
GB-Dorset DT2 8DH

Head(s) of research team(s) [name(s) and address(es)]:

Dr. D. Ramsden
Radiological & Safety Division
Atomic Energy Establishment
Winfrith, Dorchester
GB-Dorset DT2 8DH

Telephone number: 0305-63111

Title of the research contract:

Plutonium exposures in man. Direct monitoring of the lung, reassessment of the ICRP lung model and 'solubility' studies.

List of projects:

1. The direct determination of the distribution of activity within the lung.
2. Lung models.
3. In-vitro solubility studies.

Title of the project no.: 1

The direct determination of the distribution of activity within the lung.

Head(s) of project:

D Ramsden
P P Foster

Scientific staff:

K Kingman
I Pearman

I. Objectives of the project:

The overall objective of the composite programme was to improve the understanding of the behaviour of inhaled plutonium particulates in man thus increasing the realism of assessing body contents. This project concentrates on direct measurement of the distribution within the lungs. The unknown distribution of inhaled material and any changes with time are identified as major sources of uncertainty when interpreting direct measurements. Our approach was to determine the most probable distribution by using arrays of detectors and to derive a subject specific calibration factor from computer modelling, from the subjects chest structure and from studies on realistic chest phantoms. The technique is applicable at the limits of detection of the overall system.

II. Objectives for the reporting period:

The Experimental phases of the work were completed prior to 1989. The objectives of the 1989 reporting period were;

Minor modifications to the system and the interpretative techniques
Preparation of the final report

CEC funding in 1989 was minimal and this report covers the whole period 1985 - 1989 together with a brief summary of work prior to that date where it is of direct relevance to the final report.

III. Progress achieved:

Direct determination of plutonium 239 and associated radionuclides within the lung by means of external detectors rely on measuring the L-X-Rays which accompany most transuranic radioactive decays or by measuring associated low energy gamma rays. The specific emission rates of such radiations are low and the radiations themselves are easily absorbed within the chest structures. Such radiations must be detected above a background count composed of degraded low energy radiations which is also dependent on the subject's presence and on his chest structure. Detector development and application in this field, whilst still progressing, is not expected to produce a major breakthrough. Current limits of detection are such that there is insufficient sensitivity for assessing pure plutonium 239 in the human lung at levels around the annual limit of intake (ALI). However in much of the nuclear industry plutonium, often in the oxide form, consists of a mixture of the plutonium isotopes together with Amercium 241, the daughter of Pu 241. With such a mixture of radionuclides the specific X-ray emission is enhanced and this, together with the presence of 60 Kev radiations from Am 241, means that the sensitivities of current systems are just sufficient to monitor at the ALI level. The ALI, derived from ICRP 30, is recognised to be conservative and local ALIs may be derived, based on observations of that specific material in man. Such a local ALI is used by ourselves (Ref 1 of Project 2) and consequently direct monitoring of the lung is widely used in these laboratories. Detector responses have then to be interpreted in terms of the lung contents thus requiring system calibrations. The standard calibration is the response of the system to a realistic chest phantom, as developed by the Lawrence Livermore Laboratories. Corrections to this calibration are restricted to matching the soft tissue depth overlying the rib cage. The major source of uncertainty is that arising from the inhomogeneous and unknown distribution of activity within the lung. The magnitude of this uncertainty, based on measurements on men accidently contaminated with low levels of plutonium, can approach a factor of 3. This uncertainty needs to be quantified and reduced prior to full application of direct monitoring laboratory techniques.

Methodology

The methodology used in this project splits between hardware, software and the verification of the calibration factors.

a) Hardware

Hardware development was mainly done outside the current contract and is only briefly summarised here. The detectors consist of phoswich (dual phosphor) arrays above and below the chest. One array consists simply of two 12.5 cm diameter circular phoswiches whilst the other consists of six, close packed, 7.5 cm, square phoswiches. Each phoswich has its own pulse rise time 'vetoing' circuit to ensure that the only pulses analysed are those arising in the thin sodium iodide phosphor only. The arrays are then multiplexed into an ADC with in-built dead time correction, prior to analysis of all detectors by a computer based pulse height analyser. This analyser also receives pulses from an array of large sodium detectors, used to assess non plutonium activity within the subject. Module faults and the need for frequent retuning of the components in order to maintain peak operation of the system were fairly frequent and did adversely effect progress several times during the period of the contract.

b) Software

Two main computer programs were developed over the period of the project. The first, BODMOD, developed a model of a 'standard' man thorax which was then modified to match the specific subject. It is emphasised that the models are not anatomicaly realistic but are developed as a matrix of attenuation coefficients. Thus the thorax is expressed as a three dimensional regular matrix of 2.5 cm cubes. Data for each cube are attenuation coefficients at different energies for a specified mixture of soft tissue, adipose tissue, bone, lung tissue and air. The modelling of tissue boundaries can be improved by a finer cell size. The original data, for the 'standard' man, was derived from anatomy atlases, the IRCP 23 data and from the Lawrence Livermore phantom. This standard man model is them modified to match the individual subject. The modification is

basically one of identifying on the subejct certain physical characteristics eg the position of the sterrial notch and the xyphoid, the positioning and spacing of ribs from routine chest x-rays and the depths of overlying soft tissue and adipose tissue from ultrasonic scans. The standard man model is then compressed or extended to match these physical characteristics.

The second program, PECAL takes the output of BODMOD and predicts the detector response for any distribution of activity within the lung spaces and any positioning of the detectors external to the skin surfaces as a series of 'cones'. Photon flux within a cone is calculated from the solid angles and attentuation of the radiation together with modifying factors for the effects of ribs and built up by in-scatter from adjacent cones. The integrated flux at each detector is scanned over all point sources and all cones. Different distributions of activity within the lung, expressed as a sum of point sources give a series of unique ratios between detector responses.

c) Calibration factors

The theoretical responses from the computer models are regarded as relative responses only. Normalised calibration factors are obtained from the Lawrence Livermore chest phantom for uniform distribution in the 'standard' thorax. This phantom has a series of overlays to build up the chest wall thickness and a pair of 'lungs' were modified to hold point sources. A further series of studies done on simpler phantoms, were studies of tissue equivalent material with lung spaces and planar ribs. A combination of such studies was used to assess the realism of modified calibration factors generated by the computer models for inhomogeneous distribution of plutonium 239, amercium 241 and cobalt 57. The next step is the verification of the calibration factors by measurements on man. Project 2 describes studies on human volunteers who had enhanced known quantities Co 57 cobaltous oxide particles. Advantage was taken of the presence of these volunteers to validate the whole calibration approach for 120 KeV radiations. The ideal verification, measurements on man with known quantities of plutonium and amercium could not of course be done but the system was extensively tested both on a subject who had positive lung

counts from these radionuclides and on groups of plutonium workers with non-positive counts and on groups of non-exposed normals.

Results

Results for all the work on this project are detailed in the references. They are only briefly summarised here. The early work on the computer codes and on the detector systems are not summarised as their effectiveness on progress in those areas must be judged by the realism and detection capabilities of the whole system. However the 'areas of difficulty' highlighted by the computer codes are discussed briefly.

Comments on Computer Codes

The approach and code development worked well for L-X-Rays in the region 10-25 KeV where absorption losses were high. The judgement of success being the match of predicted x-ray photon flux with that observed from the phantom prior to normalisation. For higher energy radiations, 60 KeV from Am 241 and 120 KeV from Co 57 the initial predicted flux was considerably lower than that observed, demonstrating that the effect of build up factors were not being full accounted for, the effect being more pronounced at higher values of chest wall thickness and also more pronounced with material in the right lung than the left. The build-up parameters within the computer model were revised and based on experimental observation rather than theoretical values. The initial magnitude of this affect with earlier models was a factor of two, the refined model reduced the effect to a statistical one ($\pm$15%). Modifications to the computer codes aimed at further improving the determination of most probable distribution were necessary throughout the project. Gross artificial effects could occur eg positioning the plutonium activity close to the lung surface behind a rib, but in general the modifications were applied from a successful starting position of the computer codes agreeing with observations on man to within a factor of two.

The calibration factors

Summaries of results are presented in tabular form expressed as a ratio of predictions to observed responses. Each ratio is subject to appreciate and variable statistical errors. The maximum value of the statistical error was 20%. For studies on point source distribution each calibrations both observed and predicted, was the mean of at least ten point sources distributions.

Comparisons with the Livermore Phantom

a) Plutonium 239 homogeneous distributions - ratio of predicted to observed responses

Chest wall thickness (cm)		Left array	Right array
Left	Right		
0.63	0.54	1.06	0.72
0.89	0.78	0.96	0.82
1.13	1.04	1.06	0.85
1.33	1.34	1.00	0.78
1.61	1.49	0.87	0.83

Note that underprediction for the 'right array' is common for all measurements on the Livermore phantom. This underprediction is not present in point source studies nor in measurements on man, see below, it would therefore appear to arise from some "artifact" in the phantom (RH side) which the modelling programmes do not reflect. It was considered not to be productive to modify the modelling to remove this effect as the immediate secondary effect would be overpredictions in man.

b) Plutonium 239 - point sources - ratio of predicted to observed response

	Left array	Right array	Full array
Sources in left lung	1.5	0.5	1.4
Sources in right lung	0.6	1.0	1.0

Note that absolute responses vary by several orders of magnitude with changes in the relative positions of source and detector. The table summarises an extensive series of studies in a very simplified form.

c) Amercium 241 - homogeneous distributions - ratio of predicted to observed response

Chest wall thickness (cm)		Left array	Right array
Left	Right		
0.63	0.54	0.6	0.5
0.89	0.78	0.7	0.4
1.13	1.04	0.6	0.5
1.33	1.34	0.6	0.5
1.61	1.45	0.6	0.5

This illustrates the poor match of build up for modelling on this phantom. Relatively small changes in input parameters to the model can remove this effect but the current model parameters satisfactorily predict the responses from point sources and from man (see below).

d) Co 57 - point sources - ratio of predicted to observed responses

	2 Dimension Model /Buildup		3 Dimension Model	
	Left array	Right array	Left array	Right array
Left lung	0.84	0.82	0.87	1.03
Right lung	1.04	0.81	1.07	0.84

Note that build up factor modelling was not attempted for the full 3 dimensional model.

e) Co 57 - Response for man known lung content - unknown distribution

Subject	Left array	Right array
A	0.85	1.00
B	0.97	0.98
C	1.08	1.16
Mean	0.97	1.05

These results summarise a whole series of measurements with time. In particular they cover a time period up to one year during which the distribution pattern changed. They also do not show any underprediction for the Right array as is seen in responses to phantom with a homogeneous distribution, neither to they show any evidence of underprediction because of build up effects - an effect which should be greater at 120 KeV than 60 KeV. The table summarises the overall system's ability to respond to unknown distribution effects at 120 KeV. How does it respond to the lower energy radiations for plutonium and amercium?

f) Mixed transuranics radionuclides in man - unknown distribution - unknown lung content

Left Array		Right Array	
Plutonium	Amercium	Plutonium	Amercium
1.6	1.31	1.22	0.80

Presentation of predicted to observed response ratios when the lung contents are unknown is obviously not possible. However in one case there are sufficient well documented secondary indications to assess the lung contents, independently of lung counting, within the error bounds of this current work. Such secondary indications are a combination of personal air samples, nasal smears,early and late faecal samples and early and late urine samples. Naturally some modelling of overall lung clearance was necessary but as the measured lung clearance agreed with the faecal monitoring pattern and also agreed with the ICRP lung model, there is sufficient confidence in the data to present the information in the same style as the tables above.

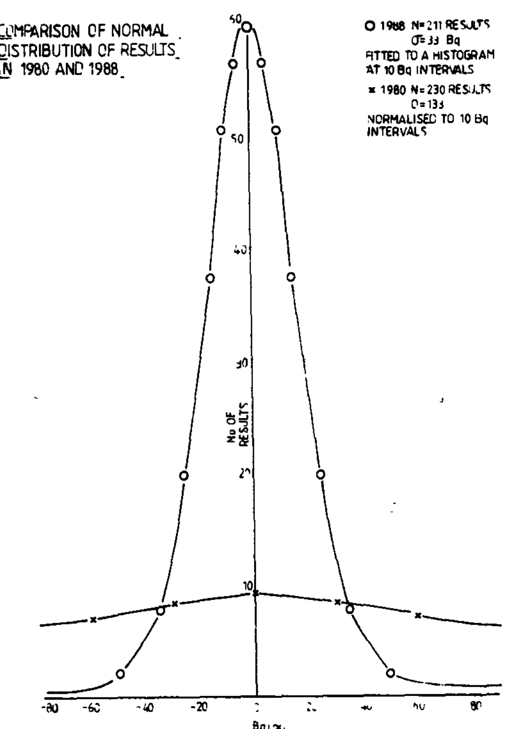
Choice of detectors

Throughout the whole of this project the primary detector has been the phoswich. During the same period there has been a move by some major laboratories within this field of work towards using liquid nitrogen cooled, high intrinsic germanium detectors specifically developed for enhanced low energy responses. The advantages of the phoswich detector are large surface area, low background and operation at room temperature. The advantages of the solid state detectors are high resolution and simper electronic circuitry. The choice between the detectors is largely a matter of judgement and cost as both approaches have comparable performances. However, in order to achieve sufficient surfaces area, solid state systems as configurated in arrays. Such array configurations

could be directly amenable to the calibration approach pursued in this project. A study was therefore carried out comparing the theoretical and experimental responses of both types of primary detector in order to see whether the phoswich approach, summarised in this project, could be bettered if the detector arrays consisted of intrinsic germanium devices. The theoretical study consisted of deriving intrinsic efficiencies to distribute point sources with a lung matrix. The experimental studies used both detectors on distributed sources with the lung of realistic phantom and included measurements on man.

The overall conclusion was that he phoswich was better in terms of limits of detection and in overall efficiency whilst the solid state detector was better at assessing discrete point sources especially if those sources, were near the lung surface. The relative responses of the two detectors, after corrections for surface area and intrinsic efficiencies, varied with source distribution. All observations could be explained by scattering outside the photopeaks and by build up effects. The phoswich detector, being of poor resolution contains an appreciable proportion of non-photopeak response whilst the solid state detector, with its high resolution can only achieve the required low background by concentrating on the photopeak region only. There was no indication that the use of solid state arrays would improve on the overall findings of this project using phoswich arrays. The converse applies in that laboratories using solid state arrays could improve their system by including inhomogeneous distribution within their calibration approach.

The final aim of the project was that it should be applicable to man in a routine way, usable at the limits of detection of the overall system and demonstrate improvements in assessment of plutonium in man. In 1980, prior to the commencement of this project data was published - IRPA Jerusalem - showing the results of monitoring programmes of about 200 measurements on plutonium workers who had no history of acute or chronic intakes. The distribution followed a normal pattern about zero with σ values 133 Bq. A similar detection pattern was produced in 1988 using the technique developed under these projects. The new pattern was still distributed around zero but the σ value has been reduced to 33 Bq. In both cases the material was taken as mixed oxide fuel with known contents of amercium 241.



Other Groups collaborating

None directly but the project is also partially supported within the UKAEA RPR progress and as such the data discussed with other laboratories working within this field in the UK.

Publications

Assessment of Plutonium in the lung
D Ramden, P P Foster
ATOM (UKAEA) Nov 1986

"BODMOD - A Computerised Chest Modelling System"
K P Kingman
AEEW RSD/TM 3 1985

"Response modelling for distributing sources in lungs"
F L Davies AEEW RSD/TM 12 1986

"The predictions of detected efficiencies in lung monitoring"
L F Alexander
AEEW RSD/TM 8 1987

"Tests on a LOAX detector'
A Cooper
AEEW RSD/TM. 1989

Title of the project no.: 2

Lung Models

Head(s) of project:

D Ramsden
P P Foster

Scientific staff:

I Pearman

I. Objectives of the project:

Within the overall objective of improving our understanding of the behaviour of plutonium particulates in man, this project covered two aspects. Firstly taking the lung Model, previously developed at Winfrith under CEC contract and expanding this model to include urinary excretion predictions. Such predictions are then compared with the data generated under project 3 of this programme. Secondly a study on the clearance of inhaled cobaltous oxide (Co57). This study, was part of a joint European interspecies intercomparison. The Winfrith involvement was studies on man, inhaling a 'standardised' aerosol. Measurements were continued so as to compare long term retention and excretion with current lung models.

II. Objectives for the reporting period:

The objectives of this reporting period were:

a) the final intercomparisons of experimental data and theoretical models on lung retention and urinary clearance in man.

b) Publication of results obtained.

CEC funding was minimal in 1989. This report summarises progress over the period 1985 - 1989.

III. Progress achieved:
Cobalt 57 Studies

The purpose of the joint EULEP study, which covered ten animal species and seven laboratories, was to test the hypothesis, central to the new ICRP lung model, that although the rates of mechanical clearance of particulates from the lung were largely independent of the inhaled material and markedly dependent on the animal species, the rates of translocation (solubilisation) of inhaled material to the blood were largely independent of the animal species. It is not the remit of this progress report to summarise the whole EULEP study and it restricts itself to measurements on man. The time-scales of the interspecies intercomparison was set by the life span of the small rodents and measurements on man, for that project, were completed at 200 days post inhalation. The measurements which then continued with for a further 2 years are reported here.

Methodology

The methodology of the study was straight forward. Four healthy non-smoking male volunteers participated, two inhaling particles of 0.8 µm (MGD) and two particles of 1.7 µm. The inhalations took place at the laboratories of AERE Harwell using proven equipment with the aerosols being generated from resuspension of the primary material generated at GSF Neuherburg FRG. Inhalation, by mouth, had an initial deposition target of 100 KBq. After an initial measurement at Harwell the subjects returned to Winfrith and were routinely measured on a calibrate body monitor for lung and whole body, Co 57 contents and monitored for urinary and faecal clearance using standard analytical techniques. Measurements continued until the levels were below the limits of detection, at 600 days for bioassay and 1000 days for lung monitoring.

Results

1 A standard protocol for expressing the results was common to all participating laboratories so that data could be compared for all species. This expressed urinary and faecal excretion rates (per day) as a fraction of the contemporary lung contents. A brief summary of this data is tabulated below.

	0.8µm MGD		1.7µm MGD	
	Subject A	B	C	D
*Range of Urinary excretion	2.0	2.0	1.0	1.0
Days 3 to 250	4.0	4.5	3.1	5.3
*Range of Faecal excretion	3.3	5.8	17.3	9.1
Days 3 to 250	0.2	0.4	0.3	0.7

*Expressed as fraction of contemporary lung contents per day x 10^{-3}

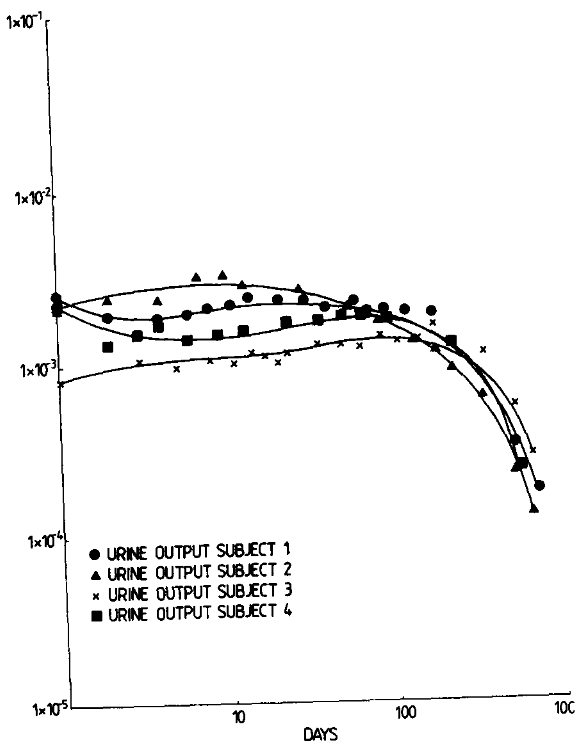
This data was then interpreted as translocation rates and mechanical clearance rates - excluding first 5 days.

	Subject A	B	C	D
*Translocation rate	0.35	0.45	0.21	0.39
(Standard Deviation)	0.07	0.07	0.07	0.07
*Mechanical clearance rate	0.04	0.20	0.07	0.10
(Standard Deviation)	0.05	0.10	0.07	0.09

*Expressed as % of day

The activity balance was monitored throughout the study and varied between 91 and 100%.

2 After the completion of the interspecies study measurements continued and are summarised in figures 1 to 3 presenting lung retention, urinary excretion and faecal excretion on all subjects up to 1000 days.


1×10⁻¹
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1×10⁻⁴
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DAYS
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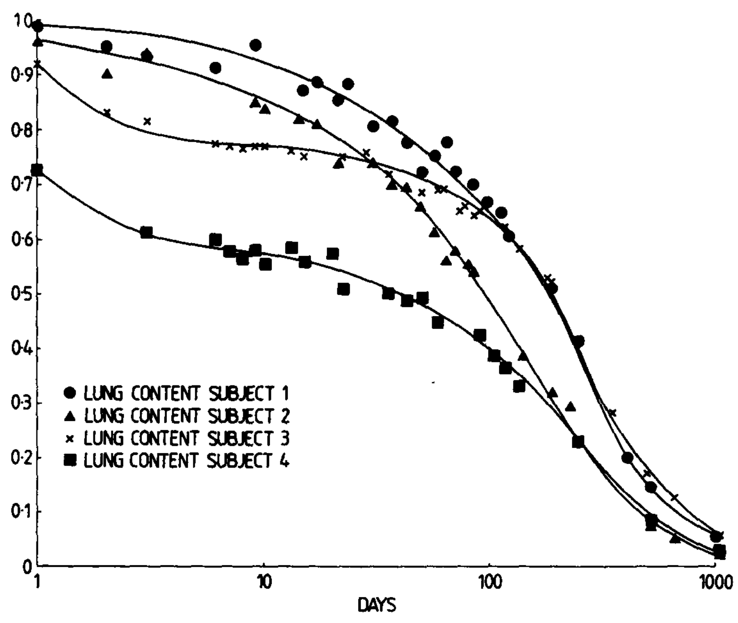
▲ URINE OUTPUT SUBJECT 2
× URINE OUTPUT SUBJECT 3
■ URINE OUTPUT SUBJECT 4


1·0
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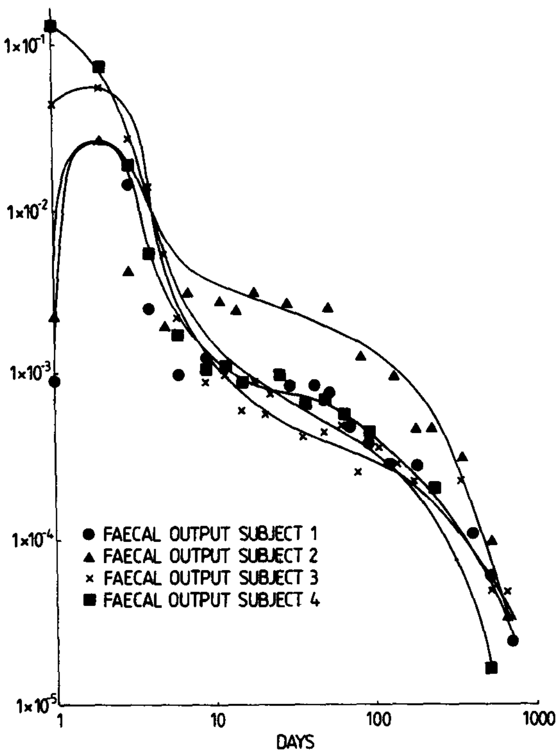
DAYS
● LUNG CONTENT SUBJECT 1
▲ LUNG CONTENT SUBJECT 2
× LUNG CONTENT SUBJECT 3
■ LUNG CONTENT SUBJECT 4



Discussion

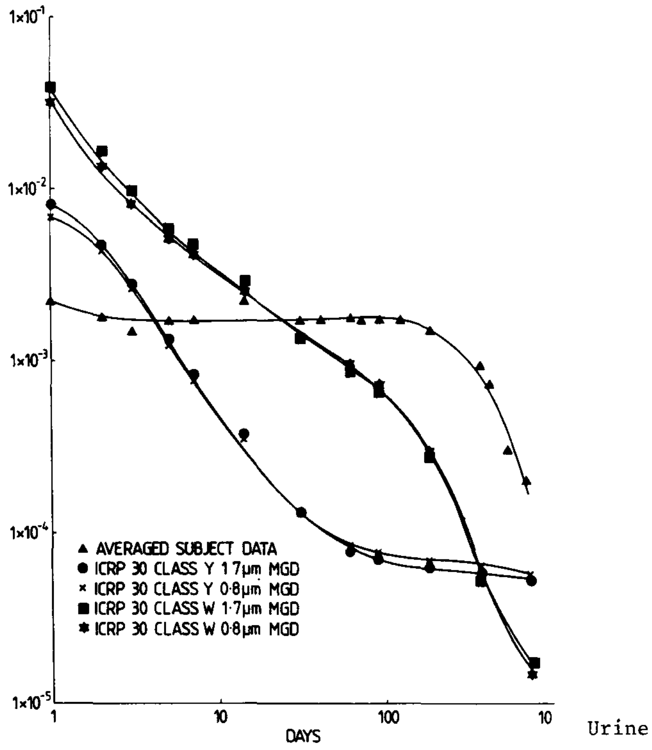
1 A short summary of the interspecies study is:-
The cobalt oxide retained in the lung of man for longer periods than other species. At day 200 the fraction of initially deposited material in the human lung was about 50% compared to 33% for the baboon, with other species varying between 1% and 15%.

For translocation to blood the rates between species were still variable but the range was narrower than that observed for mechanical clearance. For man the translocation rate was approximately 0.4% per day, independent of particle size. Other animal species gave values in the range 0.2 to 2.0% per day. The temporal pattern of clearance rate showed marked species variation with man being fairly constant and the nearest species match being obtained from the baboon.

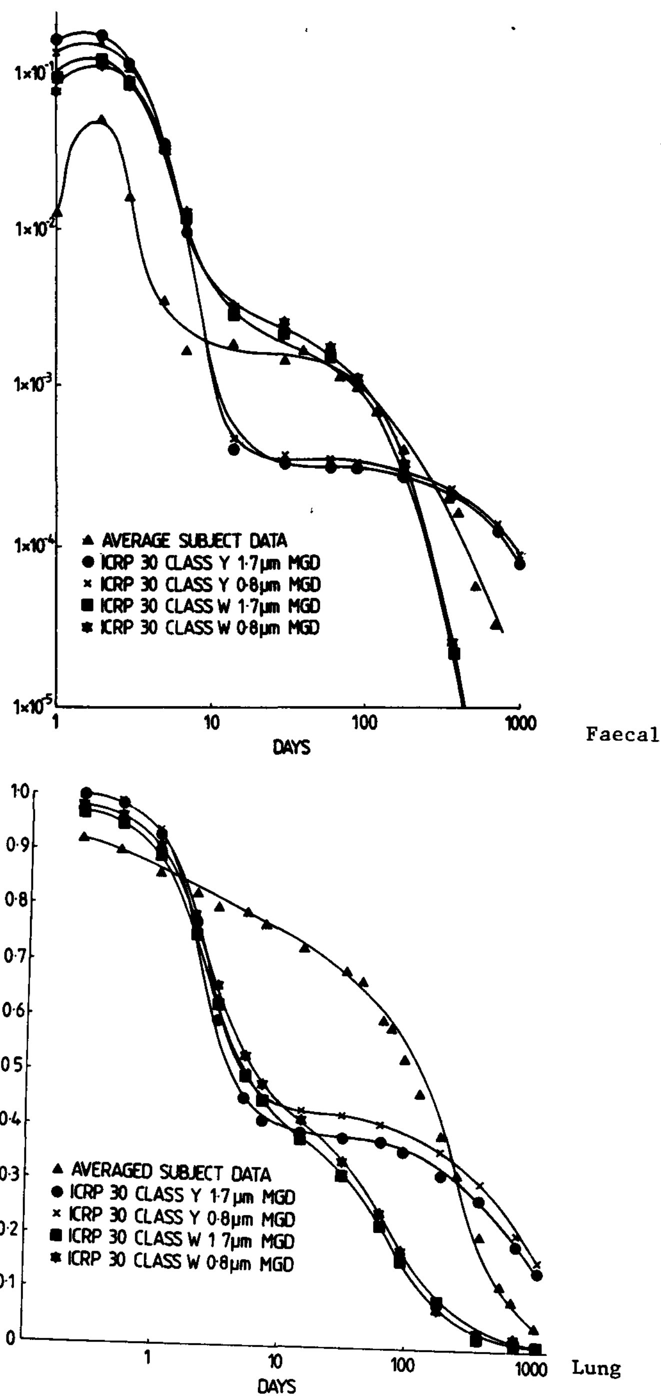
2 The long term clearance and retention data were averaged over all four subjects and compared to ICRP behaviour as illustrated in Figs 4 to 6.

Three of the four subjects lung retention data could be fitted to a two exponential pattern with short term clearances of about 1 day and long term retention of about 200 days. The other subject showed an intermediate component of about 44 days - a component which is commonly seen in measurements of accidentally inhaled particulate material in man.

Although the faecal excretion pattern is not dissimilar to ICRP class W, this behaviour is not seen in either lung retention nor urinary excretion. In particular the patterns are different over the period 20 to 500 days - periods in which routine monitoring after intakes are usually pursued to provide data on which dosimetry estimates are based. This urinary excretion pattern is also discussed below.



Urine


1×10⁻¹
1×10⁻²
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▲ AVERAGE SUBJECT DATA
● ICRP 30 CLASS Y 1·7µm MGD
× ICRP 30 CLASS Y 0·8µm MGD
■ ICRP 30 CLASS W 1·7µm MGD
✱ ICRP 30 CLASS W 0·8µm MGD
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DAYS
Faecal
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▲ AVERAGED SUBJECT DATA
● ICRP 30 CLASS Y 1·7µm MGD
× ICRP 30 CLASS Y 0·8µm MGD
■ ICRP 30 CLASS W 1 7µm MGD
✱ ICRP 30 CLASS W 0·8µm MGD
1
10
100
1000
DAYS
Lung

Lung and Urine Modelling

Methodology

The development of the Winfrith lung model was completed under CEC Contract. The model, currently in use for routine dosimetric purposes in these laboratories was not further developed until data from Project 3 was available. The model was then used and comparisons made in three areas.

1 The in-vitro solubility of industrial plutonium oxide dusts gave an excretion pattern which could be 'convoluted' with the lung model to give predicted urinary excretion patterns which could be compared with those currently used by ourselves and by other laboratories.

2 These curves were also compared with observations of Co57 in man.

3 The Winfrith lung model and associated urinary excretion functions were used in a UK laboratory intercomparison of plutonium dosimetry methodologies.

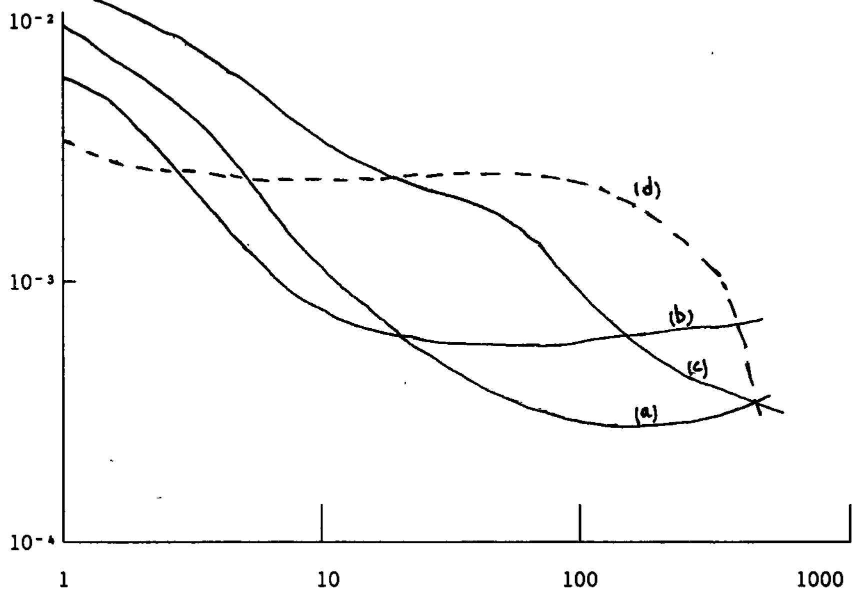
In addition the model was also 'formalised and used to derive the site specific ALIs for these laboratories as required by UK legislation.

Results

1 The comparison of model predictions and experimental data is presented and discussed in Project 3.

2 The current Winfrith urinary excretion model following inhalation is compared to data from Cs57 studies in Figure 7.

Normalised
Excretion
Rate



(a) Jones Model DAYS

(b) ICRP 54

(c) Winfrith Model

(d) Co 57

3 The UK intercomparison of plutonium dosimetry covered 4 data sets, each generated from measurements on man. For one of these data sets, intakes by inhalation with long term excretion, the model was used and the results compared to the answers from the other 6 participating laboratories. The percentage spread about the mean for all laboratories for this particular case was 43% with the predictions from this model being within 5% of the mean.

Discussion

Models for lung retention, including that developed here, can now be encompassed by the, flexibility of the new ICRP lung model. They can be successfully used for dosimetry purposes when considering both direct monitoring and faecal excretion data.

Models/predictions for urinary excretion are less securely based and still rely, to a large extent on the original Wright-Langham data. At present, beyond 1000 days post intake, this still remains the main source of data whilst, for class Y type behaviour, urinary excretion during the first few days is governed by particle size distribution, by particles shape and by traces of more soluble material. The important period for routine urine assessment for dosimetric purposes is that between 20 and 200 days (see Figure 7) and it is in this period where the major differences occur between different models. This last statement will probably still be true when the new ICRP model is published.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

GSF Neuherberg FRG

NRPB Chilton UK

CEA Brugeres-le-Chatel F

MRC Harwell UK

IGT/KFK Karlsruhe FRG

AERE Harwell UK

EEJIC Karlsruhe FRG

V. Publications:

1 Justification, calculation and use of Site Specific ALIs
D Ramsden, P P Foster - CEC Workshop
Radiation Protection Dosimetry 26 (1 to 4) 259 1989

2 An interspecies comparison of the lung clearance of inhaled monodisperic cobalt oxide particles
M R Bailey et al
J Aerosol Sc 20 (2) 169 1989

3 Lung clearance of inhaled cobalt oxide in Man
I Pearman et al
SRP Int Symposium Malvern UK 1989

Title of the project no.: 3

In-vitro Solubility Studies

Head(s) of project:

D Ramsden
P P Foster

Scientific staff:

I Pearman
M Bains

I. Objectives of the project:

The 'solubility' in man of dusts from mixed uranium/plutonium oxide fuels determines the validity of urine monitoring as a means of assessing systemic uptakes and intakes of such materials. In-vitro solubility studies have been used in these laboratories and by other workers to characterise the availability of material over time scales from a few days to a few months. This experiment was planned to extend the studies over a period comparable with the predicted main component in current lung models for class Y material ie up to three years. The in-vitro behaviour was then to be compared with the parameters in lung models and with observed behaviour in man. The final objective was to comment on the realism of the assessment of intakes from urine monitoring.

II. Objectives for the reporting period:

The experimental phase of the project was completed prior to 1988 and the current year was devoted to the analysis of the data and preparation for the final report.

This report covers the period 1985-1989 together with a brief summary of work prior to that date.

III. Progress achieved:

The experimental phases of this project started in 1983 and is described in the 1980-1984 Progress report of the CEC Euratom Radiation Protection programme. Briefly three parallel rigs were commissioned each containing a mock lung consisting of a characterised semi-permeable membrane. Each 'lung' contained about one litre of 'lung fluid' maintained at 37°C under an inert atmosphere, the fluid being remotely agitated. The outsides of the membrane were swept by 'mock plasma' at 1.5 litres per day and the plasma was collected and analysed. A suspension of particulate industrial dusts of uranium/plutonium oxide was introduced into the lungs after characterisation for activity and particle sizing. The initial characterisations are given in table 1.

Table 1 Input parameters to mock lungs

a) Chemical composition of plasmas

Chemical composition of plasmas	g/litre
Sodium Chloride	68
Ammonium Chloride	5.4
Sodium bicarbonate	22.8
Clycerine	3.8
L-Cystine	1.2
Sodium dihydrogen phosphate	1.9
Tri sodium citrate	0.6
Calcium chloride	0.1
Sulphuric acid	0.3ml
Thymol	10 ppm

b) Radioactive components

Radioactive components	Rig 1	Rig 2	Rig 3
Count medium diameter (μm)	0.4	0.75	1.18
σ g	1.7	2.26	1.58
Mass medium diameter (μm)	1.21	1.55	2.13
Total initial alpha activity (MBq)	2.02	2.02	3.0

Composition at March 1983		
(% by alpha activity)	Pu239	32.6
	Pu240	30.0
	Am241	26.9
Pu241 Total Alpha		15.0
Uranium (natural) Plutonium (by weight)		2.0

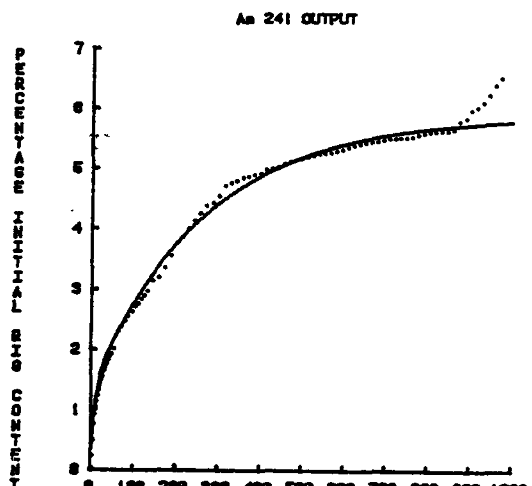
Prior to start up the membrane was characterised by electric microscopy and by a series of tests using colloidal gold-198 and indium 111. The effective pore size of the membrane lay in the region 1 to 20mm. Transferrin was added to rig 2.

The experimental phase of the study, not described here lasted until 1986 for all the rigs. The experiments were stopped just prior to the planned period of 3 years because all three rigs suddenly began to demonstrate increased transfer of activity across the membranes. This effect was coincidental with the appearance of 'bacterial growth clouding' within the plasma solutions. The bacterial growth was identified as Pseudomonas aeruginosa (pyocyarea) and tests were necessary to investigate whether the increases in membrane transfer were associated with the growth. No plutonium activity was associated with the bacteria and further tests on the membranes, repeating the initial Au-198 studies showed that the membranes were beginning to fail because of radiolytic effects. The experiment phases were therefore brought to orderly decommissioning during 1986.

Results

Data was collected as the "solubilised" fraction of material. During the initial phases of the study daily analyses were performed. The analytical period then extended to weekly and finally monthly. Samples were analysed for uranium (initially), Amercium 241 and total plutonium. the effects of the occasional failure of circulation, via peristaltic pumps, were to introduce a step change in the observed material transfer which was quickly 'smoothed out' by restoring circulation.

The data was stored as cumulative solubilised fractions and a typical output graph is shown in the Figure.



Rig No 1 Cumulative Amercium solubilised fraction as percentage of initial contents

After decommissioning the residual activities in the lung fluid and on the membrane were analysed in order to achieve a mass/activity balance.

Analysis of results

Initial analyses showed that all the outputs could be fitted to 'solubilisation' expressions containing three exponents.

$$\text{ie} \quad S = So + \sum_{1}^{3} S_n e^{-\lambda_n t}$$

with the values of λ_n lying in the ranges:

λ_1 half lifes of clearance 1 to 6 days
λ_2 half lifes of clearance 20 to 80 days
λ_3 half lifes of clearance 170 to 500 days

Any longer lived components were not evident and are included in the permanent retained fraction So.

The presence of plutonium 241, at 1.1% by weight in the original radionuclide mix, meant that the analyses for Am241 solubilities had to be corrected for the grow-in of amercium for Pu241. This was done by standard methodology. The values of So, S_n, λn were determined by best squares fitting techniques and are given in table 2 below.

Rig 1

	Uranium	Amercium 241	Total plutonium
So (as fraction)	0.875	0.958	0.973
λ^1 (d^{-1})	0.285	0.118	0.264
S_1 (as fraction)	0.122	0.013	0.012
λ^2 (d^{-1})	Not determined	Not present	0.008
S_2 (as fraction)	"	"	0.0038
λ^3 (d^{-1})	Not determined	3.79×10^{-3}	2.54×10^{-3}
S_3 (as fraction)	"	0.0329	0.0103

Rig 2 This rig contained transferrin

	Uranium	Amercium 241	Total plutonium
So (as fraction)	0.71	0.958	0.959
λ^1	0.319	0.624	0.425
S_1	0.29	0.0036	0.015
λ^2		0.0079	0.0144
S_2		0.036	0.015
λ^3		1.25×10^{-3}	3.38×10^{-3}
S_3		0.013	0.011

Rig 3

	Uranium	Amercium 241	Total plutonium
So (as fraction)	0.924	0.964	0.986
λ^1	0.251	Not determined	0.122
S_1	0.076		0.0037
λ^2		0.0318	Not determined
S_2		0.0282	
λ^3		2.73×10^{-3}	4.08×10^{-3}
S_3		0.0156	0.0107

Discussion

a) The effect of transferrin

Rigs 1 and 2 were identifiable apart from the presence of transferrin in Rig 2. Although the presence of the complexing agent, citric acid, should have ensured that any solubilised material was held in a stable state, the protein complexing agent, was added to one rig to see whether this stability did in fact exist.

There was no doubt that the amount of uranium, apparently solubilised, was markedly effected by the presence of transferrin, increasing for about 10% to 30%. There is also no doubt that the presence of transferrin had no effect on the amercium solubilised. The effects on the plutonium solubilisation were ambiguous with the total amount being 'solubilised' being 2.7% and 1.4% in the rigs without transferrin and 4.1% in the rig with transferrin. All the increased solubility occurred in the shortest component. For the purpose of comparison this shortest component (rig 2) is not included in further analyses.

b) Amounts available for 'solubilisation'.

The main test of 'realism' in the study is estimating the total amount of material which would be eventually 'transported' from the lung to the blood and hence to bone and liver. This study makes no attempt to describe the behaviour of the material once 'solubilised' - such information being available from various studies on the Wright-Longham patients, on a few well documented cases of accidental intakes and on studies on animals. The numbers generated by this study can be compared with the figures given for the ICRP lung model and for figures generated for the AEEW lung model, see Project 2.

Total activity eventually 'solubilised' from lung - (as fraction)

ICRP 19 Class Y	0.039
Class W	0.126
AEEW Model (Project 2)	0.018
This study Plutonium	0.020 ± .006
Amercium	0.040 ± 0.002

These figures support the overall realism of the study and tend to support the previous work on the Winfrith lung model where the 'reduced' solubility was based on observations of urinary clearance in man of the same material.

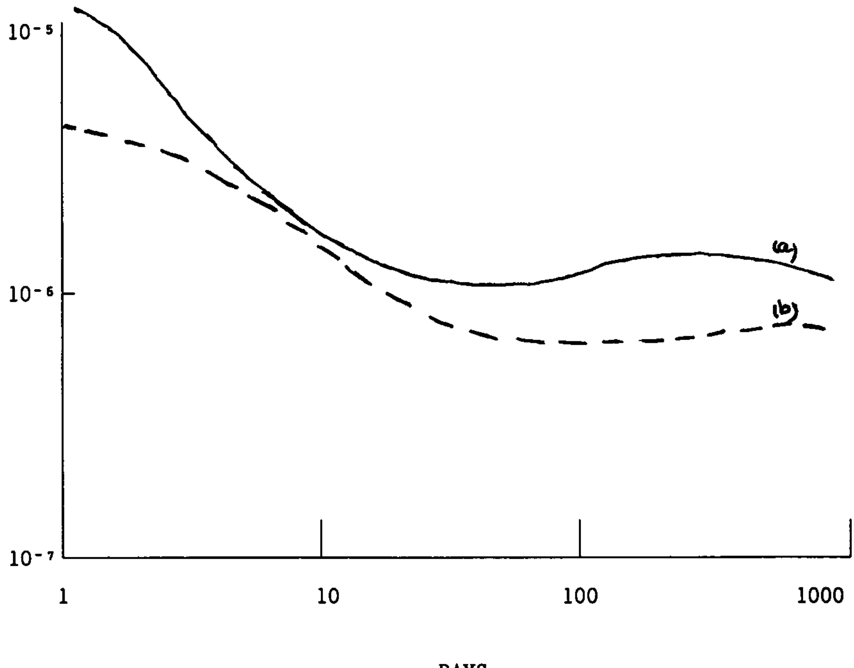
c) Time scales of clearance

Best fits to the experimental data were obtained using three components and although not all components were present in all the rigs. The following table compares the amplitudes and half lifes of the three components expressed as % of available material and also provides an 'average' figure. The shorter component, Pu/Rig 2, is not included (see (a) above) in the averages.

		Short		Intermediate		Long	
		Amp (as % of available material)	Half life (days)	Amp	Half life (days)	Amp	Half life (days)
Rig 1	Pu	44	2.6	14	87	38	273
	Am	22	5.9	- not present -		78	183
Rig 2	Pu	37	1.6	36	48	27	205
	Am	6	1.1	62	88	31	554
Rig 3	Pu	26	5.7	- not present -		76	170
	Am	- not present -		56	22	43	254
'Average'		23	3.4	28	61	49	273
c/f models							
ICRP Y		15	0.1	-	-	85	500
ICRP W		71	0.1	29	50	-	-
AEEW		32	0.1	6	30	61	300

The numbers, although supporting the Winfrith model do imply that the intermediate component is more available for solubilisation than the model would predict. All models, predict a shorter half life for the rapidly solubilised component than that observed.

d) The final comparison made here is to reflect the useability of this data in assessing plutonium intake for urinary excretion. Urine excretion models commonly used on those of Jones, Durbin of ICRP 54, based on the Wright-Langham data which are then combined with a lung model to derive urinary excretion per unit intake to the lung. The figure below, compares excretion patterns from the Jones function with the ICRP lung model to data from this study combined with ICRP class Y behaviour. The conclusion is that long term in-vitro studies can be used to predict urinary excretion and that the predicted excretion patterns agree with current models for class Y behaviour - plutonium 239.



IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

None

V. Publications:

Solubility studies on the Industrial Plutonium Dust

P P Foster, I Pearman, M E Bains and D Ramsden

Design and Interpretation of Inhalation Studies. Hanover - March 1987

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-178-B

Université Libre de Bruxelles
avenue F.D. Roosevelt, 50
B-1050 Bruxelles

Head(s) of research team(s) [name(s) and address(es)]:

Prof. J. Rommelaere
Dépt. de Biologie Moléculaire
Université Libre de Bruxelles
rue des Chevaux, 67
B-1640 Bruxelles

Telephone number: 02-358.35.30

Title of the research contract:

Cooperation between radiation and oncogenes in malignant transformation of mammalian cells.

List of projects:

1. Cooperation between radiation and oncogenes in malignant transformation of mammalian cells.

Title of the project no.:

1. Cooperation between radiation and oncogenes in malignant transformation of mammalian cells

Head(s) of project: J. ROMMELAERE

Scientific staff:
M. TUYNDER, G. HILGERS, B. AVALOSSE, Y.Q. CHEN, S. BARRIJAL

I. Objectives of the project:

This project has four main objectives :
a. To characterize phenotypic alterations which are induced by radiation in human fibroblasts and epithelial cells and which commit them into malignant progression.
b. To develop a quantitative assay for monitoring the transforming effects of ionizing radiation on human epithelial cells.
c. To identify synergistic actions between radiation and oncogenes for malignant transformation of human and murine cells.
d. To study cellular mechanisms underlying the cancer-proneness and radiohypersensitivity associated with the human syndrome ataxia telangiectasia.

II. Objectives for the reporting period:

a. To obtain a quantitative _in vitro_ cell transformation system using human keratinocytes which allows risk assessment from exposure of humans with ionising radiation.

b. To determine whether radiation transformed human cells are a preferential target for replication of and killing by autonomous parvoviruses.

c. We dealt with the molecular analysis of transient conditional response induced by radiation in normal human fibroblasts.

III. Progress achieved:

COOPERATION BETWEEN RADIATION AND ONCOGENES IN MALIGNANT TRANSFORMATION OF MAMMALIAN CELLS.

1. INTRODUCTION

The incidence of malignant transformation following exposure to carcinogens depends on several cellular parameters which may vary among different cells and/or individuals. The identification of these parameters conditioning cell susceptibility to transformation is important not only from a fundamental point of view but also for risk assessment. Cell cultures are particularly useful to this end, since their phenotype can be modulated in vitro and their progression from normality to malignancy can be monitored. The general goal of this work was to set up human cell cultures as in vitro model systems for the detection of acquired or inherited cellular changes cooperating with ionizing radiation for the induction of malignant transformation.
Human cell determinants of proneness to radiation-induced transformation are poorly understood. This is due, in part, to difficulties in quantifying in vitro transformation with cultures of human fibroblasts. We developed an alternative assay for transformation after ionizing irradiation, using cultures of human epithelial cells (see 2.1). Furthermore we have searched for cellular determinants of transformation according to a two-point strategy. Firstly, candidate human cells for an enhanced susceptibility to radiation-induced transformation include those from some cancer-prone and radiation-sensitive syndromes such as ataxia telangiectasia (AT). Cultures of cells representative of this class as well as normal human cell lines were prepared and tested for their responses to irradiation (see 2.2). Secondly, a negative determinant of malignant transformation by several carcinogens is the hypersensitivity of transformed cells to the lytic action of parvoviruses, a family of oncosuppressing viruses. On one hand, it was determined whether parvoviral replication also inhibits in vitro transformation of human cells by ionizing radiation and could provide an additional marker for the analysis of the latter process (see 2.3). On the other hand, parvovirus was used to isolate candidate transformation-modulated cellular proteins by means of their binding to the parvoviral genome (see 2.4).

2. RESULTS AND DISCUSSION

2.1. Radiation-induced transformation of human keratinocytes.

Quantitative studies on transformation following irradiation were undertaken with primary cultures of human keratinocytes. Since epithelial cells constitute a most sensitive target for radiation carcinogenesis in humans, cultures of keratinocytes may provide a valuable model system to assess the transforming risk from ionizing radiation.
As several parameters of cell transformation valuable for fibroblasts do not apply to epithelial cells, such as growth in a semi-solid medium or focus formation, a selective medium was developed permitting the identification of cell transformants. The rationale was based on the fact that cell lines established from skin carcinomas as well as keratinocyte strains transformed by SV40, human papilloma viruses (HPV) or oncogene-bearing retroviruses have been shown to express altered properties of growth and differentiation compared with normal keratinocytes (19, 20, 23, 28).

Most of these _in vivo_ and _in vitro_ transformed cells can be grown under restrictive conditions such as lowered serum or growth factor concentrations and have been shown to be more resistant than their normal counterparts to terminal differentiation stimuli (18). One of the techniques allowing the growth of normal human keratinocytes in culture consists of their incubation in serum-free medium with a low (0.1 mM) calcium (Ca^{++}) concentration but supplemented with 10 ng/ml epidermal growth factor (EGF), insulin, hydrocortisone and some other growth stimulating constituents (6, 27). Under these conditions, the single cells form colonies and do not undergo terminal differentiation. However, these keratinocytes differentiate and cease proliferating in medium with a high (1-2 mM) Ca^{++} concentration in combination with a low EGF supplement (27). These properties were used to explore the transformability of human keratinocytes after X-ray irradiation by determining their ability to proliferate in such a selective medium.
An immortalized and transformed human fibroblast line has been obtained after multiple treatments of a normal fibroblast strain with gamma-rays (17). A very similar protocol was used to obtain transformed keratinocytes. After X-irradiation (50 KV, 20 mA, 0.04 Gy/sec) the cells were incubated in proliferative medium (containing 0.1 mM Ca^{++} and 10 ng/ml EGF). 2 or 4 Gy X-rays were given to the cultures resulting in 40 and 15% cell survival, respectively. When irradiated or sham-irradiated cultures had reached 80% confluency they were split in 3 from which one dish was counted, another incubated in proliferative, and the last in selective medium (containing 1.2 mM Ca^{++} and 0.1 ng/ml EGF). Cultures in proliferative medium were irradiated again with the same dose whent they reached 50% confluency, and so on. Almost no resistant clones were obtained after single X-ray doses, but more and more emerged after multiple irradiations. Several cultures resistant to terminal differentiation were obtained after total injury of 14 Gy (7 times 2 Gy), 16 Gy (4 x 4 Gy) or (8 x 2 Gy) and 24 Gy (6 times 4 Gy). These cultures were proliferating when parallel sham-irradiated cultures had reached senescence. Thus the transformants exhibit a prolonged life-span. Two resistant cultures designated TR7201 (total dose 14 Gy) and TR8201 (total dose 16 Gy) were selected for further characterization. Both transformed cultures proliferate in the selective medium which causes normal keratinocytes to differentiate. Surprisingly, the X-ray transformed cultures did not grow well in the medium that allowed normal keratinocytes to proliferate without terminal differentiation (low Ca^{++} and high EGF concentrations). Interestingly, many squamous carcinoma-derived cells die in medium containing concentrations of EGF which are optimal for normal keratinocytes (5). Further analysis revealed that the two transformants were aneuploid and exhibited low and high expression of markers characteristic of differentiating and proliferating basal keratinocytes, respectively. In summary, X-ray irradiation of normal human keratinocytes cultures induces the dose-dependent emergence of cell clones with longer life span than normal keratinocytes. These transformants exhibit properties of cells established from carcinomas and virally transformed keratinocytes (manuscript in preparation).

2.2. Response to radiation in normal and cancer-prone human cells.

a) Recovery and mutagenesis processes in AT cells.

Conditions of abnormal sensitivity to radiation and proneness to cancer are characteristic of some hereditary syndromes in humans. Cells from

patients with ataxia telangiectasia (AT) characterized by an hypersensitivity to the lethal effect of ionising radiation and a radioresistant DNA synthesis (22) were analyzed for radiation-responses which can be monitored in vitro and might contribute to a propensity for the induction of malignant transformation.
As a first step, we have extended and substantiated our previous finding, obtained in the framework of the previous Radioprotection Program, that an AT cell line, AT5BIVA, is deficient for the expression of the enhanced reactivation (ER) response. ER takes place when the survival of a damaged virus is higher in host cells pretreated with radiation or chemical carcinogens than in untreated cells. The ER response in mammalian cells is analogous to Weigle Reactivation in bacteria, one of the SOS manifestations. Parvovirus H-1, a single-stranded DNA and nuclear-replicating virus, was used to probe ER in irradiated human fibroblasts. It has been shown that a transient and dose-dependent ER response occurs in irradiated normal human fibroblasts. In contrast, neither time-course nor dose-effect experiments revealed a significant ER of gamma-ray or UV-damaged parvovirus H-1 in X-ray or UV-irradiated AT cells (12). It was also determined whether the expression of ER is similarly deficient when monitored with the double-stranded DNA virus HSV-1 (in collaboration with Dr. A.J. van der Eb (Leiden). Time course experiments revealed a normal ER expression in X-ray or UV-pretreated AT cells, using UV-irradiated HSV-1 as a probe (13,14).
Another conditional response identified in irradiated normal human cells was denoted Enhanced Mutagenesis (EM). EM is defined as an increased mutagenesis of damaged virus in pretreated, as compared with untreated host cells. EM accompanies ER expression in some systems studied (10,15). It was therefore of interest to determine whether irradiated AT cells are able to express EM. A forward mutation assay was used to measure the fraction of mutants among the progeny of intact or damaged HSV-1 produced by mock or X-ray treated cells of normal and AT origin. In contrast with normal human cells, the AT5BTVA cells failed to achieve a detectable EM of HSV-1 upon irradiation (13). An essentially similar difference between normal and AT cells was found when the damaged temperature-sensitive single-stranded DNA virus H1TS6 was used as a probe to measure mutagenesis (14).
Altogether, the data suggest that AT cells are deficient for a component of ER which is of little importance for the rescue of damaged double-stranded DNA but which contributes to the survival of a single-stranded DNA virus as well as to the mutagenesis of both types of damaged viruses. It is tempting to speculate that these phenotypic alterations may all be traced back to a common defect related to DNA replication across damaged templates. The latter process is indeed expected to be of prime importance not only for mutagenesis but also for the survival of damaged single-stranded DNA that cannot rely on excision repair unless first converted to a double-stranded replicative form. Alternatively, irradiated AT cells may fail to produce signals that activate the latter process.
However, AT cells are proficient in ER of double-stranded DNA viruses, an error-free process which might be due to stimulated excision of lesions from duplex viral DNA.
The relevance of the reported defect of certain conditioned processes to the AT status seems double. On one hand, the aberrant conditioned response in AT cells seems to be associated with the radioresistant DNA synthesis typical of AT cells. Indeed, cell clone 67, a partial revertant of the AT5BIVA cell line, although corrected with respect to its sensitivity to X-rays induced killing, consistently retained both radioresistance of DNA

synthesis and the impairement of the conditioned response (14). On the other hand, the failure of irradiated AT cells to undergo the induction of a mutator operating on damaged virus may be correlated with the reduced number of mutations in cellular genes induced by X-ray observed by Arlett and Harcourt (1) in the same deficient cell line.

b) Biochemical relevance of cellulár responses to radiation.

The present study was undertaken to investigate mechanisms controlling the expression of genes involved in radiation recovery processes. Several studies provided evidence that interferon alpha treatment of cells promotes their recovery after irradiation (24, 29). We obtained corrborating results since we observed a synergistic effect of radiation and IFN-alpha treatment on ER of UV-damaged H-1 virus in normal human cells.
An attempt was made at investigating the latter phenomenon at the molecular level by determining the expression of four IFN-inducible genes (i.e., 2-5 A synthetase, IFI-15K, 6-16 and 1-8) in cells exposed to IFN and/or radiation. These genes may possibly be involved in cellular recovery processes or be co-regulated with the latter. Our results show that the steady-state levels of specific IFN-induced mRNAs were higher in irradiated cells compared with cells treated with IFN alone. Run-on and pulse-chase experiments provided further evidence for this posttranscriptional mode of regulation of the IFN-induced genes by radiation.
How radiation affects mRNA decay is still elusive.
However, our data suggest that the accumulation of mRNAs in irradiated cells seems primarily due to a direct control of radiation at the level of stability rather than a consequence of the inhibition of RNA processing or protein synthesis.
This effect appears to concern, in particular, transiently expressed mRNAs, raising the possibility that part of the inductive action of radiation may be ascribed to the stabilization of short-lived cellular mRNAs.
The posttranscriptional regulatory effect of radiation could have important biological implications. Control of the expression of essential inducible genes at the level of mRNA stabilization may provide opportunities for compensatory mechanisms to operate, permitting vital cellular processes to proceed during DNA repair (manuscript in preparation).

2.3. Sensitivity of radiation-transformed human cells to oncosuppressive viruses

With the object to identify other markers for monitoring the transforming effects of ionizing radiation on human cells, we determined whether the permissiveness of human fibroblasts to autonomous parvoviruses could be modulated by radiation. Parvoviruses are known to be strongly dependent on the physiological state of host cells for their replication, a property which may account for their oncosuppressive activity in vivo and their ability to destroy selectively SV40-transformed mouse cells in vitro (21). Normal human fibroblasts in culture are remarkably resistant to transformation by physico-chemical agents (11). Exposure of human fibroblasts to ionizing radiation usually results in infinite, anchorage-independent and non-tumorigenic cell strains (4, 16). It is generally accepted now that cell immortalization constitutes a major restraint to the use of normal human cells for quantitative studies on malignant transformation by radiation and chemical carcinogens (16). Therefore, we analyzed the susceptibility to the autonomous parvoviruses H-1 and MVM of the gamma-ray induced human fibroblast cell line KMST-6 in comparison with SV40- and

chemically immortalized fibroblast cell lines. Besides their immortal state, these cell lines exhibit a series of transformation characteristics such as reduced serum requirements for growth and aneuploidy (17).
The normal parental strains and their transformed derivatives took up parvovirus with similar efficiencies. Yet, the transformed cell lines expressed 10-30 times more viral transcripts and viral proteins than their untransformed progenitors. The stimulation by transformation of viral DNA synthesis was of the same amplitude, i.e., there was a parallel increase in gene expression and DNA amplification. Thus, a generalized up-modulation of viral gene expression and DNA replication was observed in the transformed versus the normal fibroblasts (2, 8, 9).
As parvoviruses are lytic viruses, it was not surprising that the greater virus replication ability in the transformed cells was associated with an enhanced killing of the latter.
Similarly, whereas normal human fibroblasts do not appear to suffer from virus infection unless high virus inputs are inoculated, gamma-ray transformed fibroblasts were highly susceptible to virus-induced killing (7, 8).
As none of the *in vitro* transformed and immortalized cell lines was tumorigenic, the latter property is not required for cell susceptibility to parvoviruses. The fibrosarcoma-derived human cell line HT1080 ranged among the *in vitro* transformants with respect to susceptibility to parvovirus H-1 and MVM replication and killing (8).
This suggests that the acquisition of the malignant state does not add to the cell susceptibility of the SV40 and physicochemically immortalized and transformed fibroblasts. The question whether the acquisition of immortality is associated with susceptibility to parvoviruses could be answered negatively, at least for immortalization caused by SV40 (7).
Altogether, the result show that cell sensitivity to parvoviruses may constitute a marker of a preneoplastic transformed state which does not necessarily include immortality. The gamma-ray induced human fibroblast cell line KMST-6 behaves like viral and oncogene-transformed infinite or immortal fibroblasts with respect to their increased susceptibility to the replication of the autonomous parvoviruses H-1 and MVM compared with parental untransformed fibroblasts.

2.4. Detection of transformation-sensitive cellular proteins by their binding to DNA of oncosuppressive parvoviruses.

As the replication of the parvoviruses is associated with the transformed state (see above) cellular markers of transformation were sought with their help. Cellular proteins specific for the normal or transformed state may exist and exhibit specific affinity to parvoviral nucleic acids. Cellular proteins with specific affinity for parvoviral DNA were searched using the south-western blotting technique. In short, cellular proteins were fractionated by SDS-polyacrylamide gels, blotted into nitrocellulose filters which were incubated in the presence of radiolabelled parvoviral DNA fragments. By this technique a nuclear parvoviral DNA-binding protein of approximately 105 KDa molecular weight was detected that was more abundant (or more active) in the normal human fibroblast strains KMS-6 and MRC-5 than in their gamma-ray and SV40-transformed derivatives KMST-6 and MRC5VI (3).
The greater availability of the 105 KDa protein in parvovirus- resistant fibroblasts was confirmed in a series of hybrid clones between normal human- and transformed mouse fibroblasts,exhibiting varying susceptibilities to the killing effect of parvovirus MVM (3). It is tempting to speculate that the 105 KDa species is a DNA-binding suppressor protein which is inactivated, lost or repressed in transformed and parvovirus-susceptible cells. A parallel may

be drawn between such properties and those of the products of anti-oncogenes (25).

3. CONCLUSIONS

a. After repeated treatments of human foreskin keratinocytes with X-rays, cell clones emerged which resisted to a greater extent to terminal differentiation stimuli than normal keratinocytes. These X-ray transformed cultures are induced in a dose-dependent fashion, exhibit a prolonged life-span, are aneuploid, and stain positively and negatively for markers of proliferating basal and differentiating keratinocytes, respectively.

b. Cells from patients with the cancer-prone ataxia telangiectasia syndrome are altered for a radiation-induced process which plays a minor role in the reactivation (ER) of a double-stranded DNA virus but which contributes to the survival of a single-stranded DNA virus as well as to the mutagenesis of both types of damaged viruses. The data may be explained by an impaired capacity of AT cells for replication damaged viral DNA or by their failure to produce signals that activate the latter process. This deficiency seems to be associated with the radioresistant DNA synthesis and the hypomutability typical of AT cells. Our data suggest that part of the inductive action of radiation may be ascribed to the stabilization of short-lived cellular messenger RNAs.

c. Human fibroblasts immortalized and transformed by gamma-radiation were more susceptible to parvovirus replication and killing than the normal parental cells. As the radiation- transformed cells were not tumorigenic, cell susceptibility to parvoviruses may constitute a preneoplastic marker of cell transformation. A 105 KDa cellular protein was identified by its specific binding to parvoviral DNA. This protein is more abundant (or more active) in normal than in transformed human cells.

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- Hilgers, G., P.J. Abrahams, Y.Q. Chen, R. Schouten, J.J. Cornelis, J.E. Lowe, A.J. Van der Eb and J. Rommelaere (1989). Impaired recovery and mutagenic SOS-like response in ataxia telangiectasia. Mutagenesis, 4, 271-276.
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RADIATION PROTECTION PROGRAMME
Final Report

Contractor:

Contract no.: BI6-D-103-I

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Title of the research contract:

Radiation carcinogenesis in animals: search for and role(s) of oncogenes.

List of projects:

1. Radiation carcinogenesis in animals: search for and role(s) of oncogenes.

Title of the project no.: B16-D-103-I

Radiation carcinogenesis in animals: search for and role(s) of oncogenes.

Head(s) of project:

G.B. Rossi

Scientific staff:

S. Pulciani, F. Belardelli, L. Santodonato, E. Parlanti.

I. Objectives of the project:

Studies over the last few years have provided evidence that several cellular genes may exhibit potential transforming activity (proto-oncogenes) (1). Activated proto-oncogenes have been detected into spontaneous or induced animal tumors as well as into human neoplasias (1,2,3).

Spontaneous, x-ray-induced and virus-induced murine tumors have been chosen as working models to investigate the molecular mechanisms involved in the acquisition of the malignant tumor cell phenotype. Our major purpose consisted in determining the role(s) of activated proto-oncogenes in the onset and progression of the neoplastic process.

II. Objectives for the reporting period:

1) Search for and role(s) of oncogenes in spontaneus and X-ray- induced mouse tumors. By DNA-mediated gene transfer protocols we searched for trasforming genes in spontaneous reticulum cell sarcoma (RCS) of SJL/J mice (4,5), as well as in x-ray induced lymphomas in (C57/Bl/CnexC3H/Cne) F_1 mice(6).

2) Studies on the mechanisms involved in the tumorigenic behaviour of Friend leukemia cells (FLC): effects of X-ray radiation. We analyzed the expression of histocompatibility antigens and the role of activated oncogenes in the metastatic process in the FLC system (7,8,9,10). Moreover we analyzed the effects of sublethal doses of x-rays on the FLC *in vivo* behaviour.

III. Progress achieved:

MATERIALS AND METHODS

Mice. Male and female DBA/2 mice were obtained from Charles Rivers s.p.a.(Milan, Italy). SJL/J female mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Tumor cells. Spontaneous RCS were obtained in SJL/J mice at ages of 9-12 months. The RCS transplantable tumors were maintained *in vivo* by serial passage of 10^7 viable cells i.p. into 6-12 week old SJL/J mice. RCS cell suspensions were obtained by excising tumors, spleens and mesenteric lymph nodes 8-10 days after inoculation and gently teasing the tissue through nylon wool filters, as previously described (11).

Interferon α/β resistant 3C18-FLC (7) were cultivated in RPMI medium supplemented with 10 per cent fetal calf serum (FCS) (*in vitro* passaged FLC) or passaged by weekly intraperitoneal (i.p.) inoculation of 5-6-week-old DBA/2 mice (*in vivo* passaged FLC). WGA-resistant (WR) 3C1-8 FLC were isolated from *in vivo* passaged 3C18-FLC, as described elsewhere (8). A cloned WR FLC population (i.e., W2 clone) (8) was used in the experiments described in this report. The origin of IFN-sensitive *in vitro* or *in vivo* passaged 745 FLC have been previously described elsewhere (9).

RNA extraction and analysis. Total cellular RNA was extracted according to the quick phenol guanidin method (12). 20μg of RNA were fractionated by electrophoresis on 1.3% agarose gel and blotted onto nylon membrane (High-Bond, Amersham) overnight. Northern blots were hybridized at 42°C in 1M Nacl, 10% dextran sulphate, 1% SDS and 50% formamide, or 40% formamide. Following hybridization with either nick translated or random primed probes, all the blots were first soaked for 5 minutes in 2x ssc (1xssc 0.15M NaCl, 0.015M sodium citrate) and successively washed 30min in 2x ssc, 0.5% SDS at 65°C. The filters were exposed with x-ray films at -80°C using kodak cassettes provided with lanex intensifying screens.

Molecular probes. The DNA probes used in the hybridization experiments were: 1.5-kb Cla 1, Eco RI fragment from human

c-myc; 0.6-kb Hind III, Bam HI fragment cloned from BALB murine sarcoma virus and representative of mouse c-h-ras, and a 1.0 kb Eco RI fragment excised from clone phihi 3 representative of murine c-k-ras (10).

Evaluation of the number of hemoglobin-producing cells.

Percentages of hemoglobin-producing (B^+) cells were determined by the wet benzidine staining method of Orkin et al., (1975) (13).

Isolation of high molecular weight DNA. Cells were lysed by addition of 10 mM Tris-HCl, 150 mM EDTA (pH 7.8), and 0,5% sodium dodecyl sulfate and the lysates were incubated overnight at 37°C in the presence of Proteinase K (200 μg/ml). Partially deproteinized nucleic acids were extracted once with one volume of phenol, one volume of phenol/ chloroform/ isoamylalcohol (25/24/1), and one volume chloroform / isoamylalcohol (24/1). The DNA was precipitated with 2.5 volumes of cold ethanol (-20°C), dried and stored at 4°C in sterile 10 mM Tris-HCl, 1 mM EDTA (pH 7.4) (3).

DNA Blotting Analysis. Twenty micrograms of high molecular weight DNA were digested with appropriate restriction endonucleases and applied to horizontal 0.6% (w/v) agarose gels. Samples were electrophoresed at 30 V for 20 h, blotten onto nitrocellulose sheets, and hybridized for 48 h to $2x10^7$ cpm of the corresponding nick-translated (^{32}P)-labeled DNA as described by Southern (1975) (14).

FACS analysis. Cellular DNA content was assayed by propidium iodide staining method. Briefly, cells were fixed in 70% ethanol, 15 min at 4°C, washed and resuspended (10^6 cells/ml) in 1 ml of PBS, containing 0.1% NP40, additionated of 100 Kunitz units of pancreatic RNAse (Sigma, St. Louis, MO) and incubated for 10 min at RT. 5 μgr of propidium iodide (Sigma) were then added to each sample (stock solution 0.5 μgr/ml propidium iodide in H_2O, 10 μl/sample). Cytofluorimetric analysis was carried out in FACS can instrument (Becton-Dickinson, Mountain View, CA) (10).

Transfection Assays. Transfection assays were carried out by the calcium phosphate precipitation technique of Graham and

van der Eb (15) as modified by Wigler et al. (16). Donor DNA (40 μg) was resuspended in 1 ml of 0.25 M $CaCl_2$ and gently mixed with an equal volume of 250 mM 4-(2-hydroxyethyl) -1-piperazine-ethanesulfonic acid (pH 7.1) and 1.5 mM sodium phosphate. The DNA was allowed to precipitate for at least 1 h at room temperature and then added to 10 cm Petri dishes, where 1.5 x 10^5 recipient cells (NIH-3T3 or F2408) had been seeded the day before (3). NIH-3T3 mouse fibroblasts (17) and F2408 rat fibroblasts were selected for their best morphology and maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum.

DNA cloning and sequencing. DNA cloning has been performed in ZAP vector following manufactury protocols (Stratagene). DNA sequencing has been performed following the chain-termination DNA protocol described by Sanger (18).

X-ray treatment of FLC. The experiments on X-ray-induced effects on survival and mutation index have been carried out using the microtiter plate test method (19). 10^5 cells/ml were irradiated as described below and a suitable FLC dilution was immediately plated for testing cell survival. The remaining cell suspension was subcultured every 48 hrs for 8 days. After 10 days of incubation, FLC were counted and the corresponding survival was calculated. FLC samples showing a surviving fraction of 0.5 were used for the in vivo experiments. The cells were suspended in medium at the concentration of 2×10^6 cells/ml and 0.2 ml were injected i.v. in DBA/2 mice. The irradiation was performed by means of an X-ray apparatus operating at 200 KV 15 mA, with a 0.2 mm copper filter with a dose-rate of 1.2 Gy/min. The dose was delivered as single or fractionated, with different times (1 to 3 hours) between the two doses.

RESULTS and DISCUSSION

We selected three different model systems to investigate at molecular level the mechanisms of proto-oncogenes activations and their roles in the onset and development of neoplasias and during tumor progression: a) X-ray-induced

lymphomas in (C57 Bl/Cne x C3H/Cne) F1 hybrid mice (6); b) Spontaneous mouse reticulum cell sarcomas (RCS) (4,5); c) Non metastatic and highly metastatic Friend Leukemia cells (FLC) (7-10).

1) Studies on x-rays-induced lymphomas (C57 Bl/Cne x C3H/Cne) F1 hybrid mice have been irradiated with 6 Gy of x-rays, according to Covelli et al (6). DNAs from the physically induced lymphomas have been isolated and utilized to transform NIH/3T3 mouse cells in transfection experiments. 13 lymphomas have been tested and none of them has been scored positive for foci formation. These results indicate that no oncogenes, (at least detectable by means of our experimental approaches) were activated in these lymphomas.

2) Studies on spontaneous RCS in SJL/J mice. High molecular DNA was extracted from three transplantable cell lines established from spontaneous RCS of SJL/J mice. Such DNA was tested in transfection experiments. Round foci with an distinguishable morphology could be scored after 20 days in NIH/3T3 transfected cells. Several foci were selected and the established clones were further characterized (20). Filtered culture media from different clones of NIH-3T3 cells, transformed by the RCS tumor DNAs, have been tested in infection assays on normal NIH-3T3 cells. In these experiments, multiple foci of transformed cells were found. These foci showed the same growth characteristics as those obtained in the transfection experiments. These results demonstrated that the transforming activity of the RCS tumor DNAs was associated with the presence of a transforming virus.

DNAs from selected clones of NIH-3T3 transformed by the RCS virus were digested with several restriction enzymes and analyzed on agarose gels along with normal NIH-3T3 DNA. In the DNA extracted from the NIH-3T3 transformed clone, V2-6-1 specific sequences were found to be present in a high copy number. Upon digestion of V2-6-1 DNA with Eco RI, followed by gel electrophoresis in buffer with ethidium bromide, a specific DNA band of 5.6 kb could be detected, whereas no band could be detected in control DNA. We postulated that

these genetic sequences represented the genome of RCS virus detected by the biological assays. Therefore, we cloned the 5.6 kb Eco RI fragment. Then we have performed the DNA sequencing of the cloned virus by the chain-termination DNA protocol described by Sanger (18). These experiments showed that the cloned virus was highly homologous to the mouse Polyoma viruse DNA (unpublished results).

3) Studies on metastatic and non metastatic FLC variants.

Background. In previous studies we had showed that *in vitro* passaged Friend Leukemia cells (FLC) were not tumorigenic when first injected i.p. or i.v. in syngeneic DBA/2 mice (9). By repeated i.p. passages of FLC in DBA/2 mice we selected FLC variants capable of forming hemorrhagic ascites with high efficiency and of metastasizing to the spleen and to the liver, when injected either i.v. or s.c. (9). The acquisition of the FLC metastatic phenotype paralleled the appearance of a particular membrane glycoprotein pattern with respect to the non metastatic FLC (8). Using such well characterized experimental system, it was of interest to analyze some possible changes in gene expression between the metastatic and non metastatic FLC and the effects, if any, of X-ray-treatment on the FLC phenotype.

3-a. Analysis of the histocompatibility antigen expression.

An ensemble of experimental studies indicates that the expression of histocompatibility antigens on the tumor cell membrane can play important roles in controlling the metastatic growth of several murine tumors (21). Therefore we measured the levels of expression of hystocompatibility antigens on the cell membrane and their gene expression in non-metastatic and in highly metastatic FLC. Highly metastatic *in vivo* passaged FLC (either interferon-sensitive 745 or interferon alpha/beta -resistant 3C1-8 cells) expressed higher levels of class I H-2K and H-2D antigens on their cell membrane with respect to the non-metastatic *in vitro* passaged counterparts. The increased expression of H-2 class I antigens was associated with an increased accumulation of H-2D and H-2K m-RNAs (22).

3-b. Analyses of the oncogene expression. It has been widely demonstrated that oncogene activation is involved in at least some human spontaneus tumors (2,3). In tumor cell systems, a direct correlation has been shown between transformation and enhanced oncogene expression and/or mutation and rearrangment (23-25). Moreover in the FLC system, it has been shown correlations between oncogene expression and DMSO-induced differentiation (26). In the light of all these data, it seemed of interest to analyze the possible correlations between the expression of some oncogenes (i.e., c-myc, H-ras and K-ras) and the *in vitro* and *in vivo* behaviour of FLC. High levels of c-myc oncogene mRNA were expressed in all the FLC variants; no major variations in the c-myc expression were observed in FLC cultivated in medium supplemented with different FCS concentrations and/or seeded at various cell densities. In addition, no changes in the expression of H-ras or K-ras were observed between the different FLC types. We conclude, therefore, that other cellular genes, different from c-ras and c-myc, may be involved in the clear-cut differences in the FLC tumorigenic behaviour.

3-c. Effects of x-ray treatment on the FLC phenotype. The aim of this study was to investigate the effects of x-ray sublethal doses on the FLC phenotype. For this purpose both highly metastatic and non-metastatic FLC variants have been employed. The cells were grown and maintained in RPMI-1640 medium supplemented with 10% FCS. The possibility that the irradiation procedures could result in some changes in the tumorigenic FLC phenotype has been investigated according to the following working hypothesis:

Metastases to the liver and/or spleen (expected results)

FLC type	non irradiated FLC (control)	irradiated FLC
in vivo p.	+	-
in vitro p.	-	+
WR FLC	-	+

Preliminary experiments have been carried out in order to find out the doses capable of determining 50% survival with all clones, as described in the Materials and Methods. FLC samples, treated with 3 hour time interval between the two semi-doses, were injected i.v. in DBA/2 mice (10^6 cells/mouse). For the experiments concerning the in *vivo* passaged 3C1-8 FLC, the mice were injected with the following cell samples: 1) non-irradiated FLC; 2) FLC irradiated with a 2.4 Gy single dose; 3) FLC irradiated with 1.2 + 1.2 Gy fractionated dose. The mice were kept under control for survival and the dead mice were examined for metastases: no significative change in the tumorigenic behaviour between irradiated and non irradiated FLC was detected.
Similarly, *in vitro* passaged FLC were irradiated with a single dose (1.8 Gy), or with a fractionated protocol (0.9 + 0.9 Gy) and subsequently injected, as in the previous experiment. 12 days after injection, three mice from each group were sacrificed and the liver and the spleen removed. The FLC recovered from these organs were cultured *in vitro*, cloned and tested for their growth capacity in agar (9). Other mice were observed for survival. The results did not show any significant change in the *in vivo* and *in vitro* behaviour of the treated cells *vs* untreated control FLC.
Another set of experiments was performed with the WGA-resistant non-metastatic FLC; 10^6 unirradiated cells, or FLC irradiated with either a single 1.8 Gy dose or a fractioned 0.9 + 0.9 Gy dose were injected as previously described. 14 days after injection, 3 mice from each group were sacrificed and liver and spleen were removed. Neither liver nor spleen metastases were observed by gross examination. Cells obtained from these organs have been seeded in culture to detect the possible presence of small numbers of FLC. No FLC were recovered from the liver, whereas some FLC were detected from the spleen. These FLC have been grown *in vitro* and tested for WGA resistance, according to the procedure described elsewhere (8). The FLC isolated from the spleen of the treated mice maintained the WGA-resistant

phenotype. These clones were then injected i.v. into DBA/2 mice; no significant differences in the survival time have been detected as compared to mice injected with unirradiated 3C1-8 WR FLC.

Effect of X-ray radiation on the tumorigenity of WR FLC

Treatment	WGA phenotype	Mean day of death	% of mice with FLC recovered from the spleen
none	resistant	35	33%
acute dose	resistant	36	100%
fract.dose	resistant	37	100%

FLC derived from the spleen of mice injected with irradiated WR FLC were subsequently injected i.v. in DBA/2 mice, in order to check whether these FLC had acquired a more potent tumorigenic potential as compared to the control WR FLC. However, no significant change in the tumorigenic behaviour has been detected between the different WR FLC types (data not shown). Altogether these data demostrated that the *in vitro* treatment of FLC with X-ray sublethal doses does not result in any clear-cut alteration of the tumorigenic phenotype of tumor cells in the FLC system.

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V. Publications:

Pulciani S., Sakano T., Ohnishi K., Anastasi A.M., Pecorelli A., Fiorucci G., Oppi C., Rossi G.B. and Bonavida B. Detection of a transforming gene in spontaneous reticulum cell sarcoma of SJL/J mice: genetically linked and host-dependent neoplasia. Cancer Research **47**, 523-526, 1987.

Ferrantini, M., Pulciani, S., Proietti E., Lespinats, G., Anastasi, A., Ciolli, V., Rizza, P. and Belardelli, F. Studies on the expression of H-2 antigens in non-metastatic and highly metastatic Friend erythroleukemia cells: correlation with the *in vivo* behaviour of tumor cells. Clin. Expl. Metastasis, **7**, 609-625 (1989)

Sala, A., Benedetto, A., Elia, G., Pulciani, S., Ciotta, C., Parlanti, E., Santodonato, L. and Belardelli F. Studies on the possible correlations between biologic properties and oncogene expression in metastatic and non metastatic Friend Leukemia Cells variants (submitted for publication).

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-236-D

Gesellschaft für Strahlen- und
Umweltforschung mbH
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Title of the research contract:

RBE-values of monoenergetic electrons in the range of several 100 eV to 10 keV for cell transformation.

List of projects:

1. RBE-values of monoenergetic electrons in the range of several 100 eV to 10 keV for cell transformation.

Title of the project no.:

RBE-values of monoenergetic electrons in the range of several 100 keV to 10 keV for cell transformation.

Head(s) of project:

Priv. Doz. Dr. D. Frankenberg

Scientific staff:

Priv. Doz. Dr. D. Frankenberg
Dr. M. Frankenberg-Schwager

I. Objectives of the project:

RBE-values of electrons of definite energies will be determined for cell transformation using the immortalized mouse embryo fibroblast cell line C3H10T1/2. Monoenergetic electrons are produced within cells by irradiation with characteristic ultrasoft X-rays which are generated by bombarding suitable targets with accelerated protons. The radiations used are ^{60}Co gamma-rays as reference radiation and Al_K (1.5 keV) and C_K (0.278 keV) characteristic X-rays.

II. Objectives for the reporting period:

Determination of the transformation frequency in C3H10T1/2 cells for ^{60}Co gamma-rays as refenence radiation.
Determination of the OER-value for transformation of C3H10T1/2 cells.
Evaluation of the DNA lesion(s) involved in radiation-induced cell transformation by comparing RBE- and OER-values for DNA lesions and cell transformation.

III. Progress achieved:

Introduction

Cytogenetic and epidemiological studies suggest that DNA rearrangements are the main cause for cancer induction (1,2,3,4). Evidence derived from several sources implicates the DNA double-strand break (DSB) in DNA rearrangements (5,6,7).
The comparison of RBE- and OER-values for both the different radiation-induced DNA lesions and cell transformation is a useful tool to elucidate the role of primary DNA lesion(s) in cell transformation. RBE-values of densely ionizing radiations for cell transformation have been determined by several authors. RBE-values of alpha particles are in the range of about 3 (high doses) up to about 12 (low doses) (8,9,10,11). Similarly, neutrons (12,13) and ions of high LET (14) exhibit RBE-values higher than one. RBE-values of densely ionizing radiations significantly higher than one are also observed for the induction of DSB, suggesting that DSB might be a critical primary DNA lesion for cell transformation. A further support of this hypothesis may be obtained by comparing the OER-value of low LET radiations for cell transformation and DNA lesions.
Whereas the OER-values for most DNA lesions are known, the data on OER-values for cell transformation are scarce (15).

Materials and Methods

The C3H10T1/2 mouse-embryo fibroblast system developed by Reznikoff et al. (16) was used. Cells were kindly donated to us by Dr.L.Hieber, Würzburg. Cells were maintained in Eagle's basal medium supplemented with 10% heat inactivated fetal calf serum (Boehringer), 50 units/ml penicillin and 50 µg/ml streptomycine. Cells of passage 12 were incubated in 75 cm^2 flasks at 37°C in a humidified gas atmosphere (95% air, 5% CO_2). They were subcultered in 25 cm^2 flasks for 24 h and irradiated in exponential growth phase at a density of about 10^4 cells/cm^2. After irradiation cells were trypsinized, pooled, and cell concentrations determined using a Coulter Counter.
For exposure under anoxic conditions exponential cells were trypsinized and pooled prior to irradiation in suspension at a cell concentration of 10^5 to 10^6 cells/ml in glass vessels. Anoxia was achieved within 5 minutes by gassing the suspension with 95% nitrogen (< 10ppm O_2) and 5% CO_2.
Irradiations were performed with ^{60}Co gamma-rays at a dose rate of 0.7 Gy/min under aerobic and of 2.5 Gy/min under anoxic condition as determined with the Fricke dosemeter.
For the survival assays appropriate dilutions were chosen to obtain about 80 viable cells per 25 cm^2 flask. After incubation for 14 days and staining with 10% giemsa, colonies with more than 50 cells were counted as survivors. For the transformation assays, about 300 viable cells were plated in 25 cm^2 flasks. Cells were incubated for 6 weeks. After two weeks of incubation the medium was changed every week.

For the determination of transformed cells, the cultures were washed with phosphate buffered saline, fixed with methanol and stained with 10% Giemsa. Foci of type 2 and 3 were scored as transformed cells (16).
The plating efficiency of control cultures both under aerobic and anoxic irradiation conditions was 20 to 30 % . Control experiments showed that trypsinization of cells before irradiation has no effect on the radiation response.

Results

In figure 1 are shown the survival curves of C3H10T1/2 cells after γ-irradiation under aerobic and anoxic conditions. The OER for cell inactivation is approximately constant in the dose range used and amounts to 2.8.

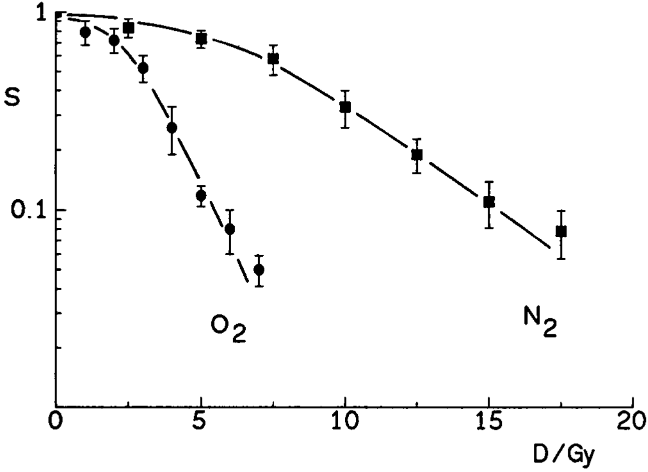

Fig. 1: Inactivation of C3H10T1/2 cells by ^{60}Co gamma-rays under aerobic or anoxic conditions.

In figure 2 the number of transformants per 10^4 survivors is plotted as a function of absorbed dose D. In addition, the mean number of dsb per cell induced under aerobic and anoxic conditions is given. The slopes of the three straight lines indicate the proportionalities to D, D^2, and D^3 of the number of transformants. For high doses the number of transformants is proportional to approximately D^3. For doses

smaller than 2 Gy (aerobic conditions) or 7 Gy (anoxic conditions), corresponding to about 80 DSB per cell, a linear relationship between the number of transformants and dose becomes predominant.

At high doses, the OER is 2.9 and decreases gradually to about 1.5 in the low dose region.

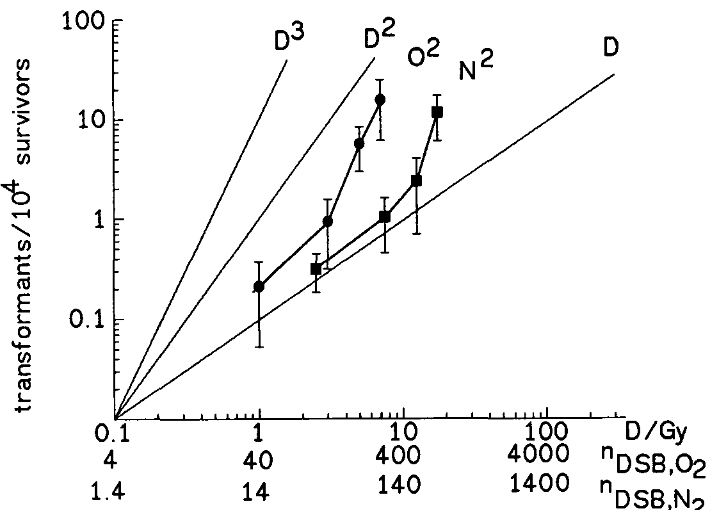

Fig. 2: Transformation frequencies after irradiation with ^{60}Co gamma-rays under aerobic or anoxic conditions. In addition to absorbed dose, the abszissa shows the mean number of DNA double-strand breaks induced either under aerobic or under anoxic conditions (n_{DSB,O_2} ; n_{DSB,N_2}).

Discussion

The inactivation of C3H10T1/2 cells by ^{60}Co gamma-rays under aerobic conditions is in excellent agreement with that found by Hieber et al. (10). The OER-value of 2.8 determined for the inactivation of C3H10T1/2 cells lies in the range of OER-values observed for most mammalian cell lines.

The transformation frequencies under aerobic irradiation conditions are in agreement

with the data obtained by Hall and Hei (9) and Hieber et al. (10) at high doses (5 Gy and 7 Gy). However, at low doses (1 Gy and 3 Gy) our data show significant discrepancies compared to the data of Hall and Hei (9) and Hieber et al. (10). This might be partially due to insufficient statistics of the experimental data. The OER-value for transformation of C3H10T1/2 cells is dose-dependent; at high doses (D> 5 Gy, aerobic condition) the OER is 2.9 and decreases gradually to about 1.5 for doses smaller than 2 Gy (aerobic conditions). These results are in contrast to those reported by Borsa et al. (15) who found a dose-modifying OER-value of 3.0 for the same cells irradiated in exponential growth phase with 250 kV X-rays. However, they found transformation frequencies which are by a factor of at least 15 higher than those observed by Hall and Hei (9), Hieber et al. (10) and in this paper.

Table 1:

Comparison of RBE- and OER-values for transformation of C3H10T1/2 cells and for the different DNA lesions.

Endpoint	RBE of densely ionizing radiations	OER of sparsely ionizing radiations
Cell transformation	> 1	1.5 (low dose region) 2.9 (high dose region)
Single-strand breaks	< 1	3
Base damage	< 1	1
DNA-protein crosslinks	—	< 1
Bulky lesions	—	—
Induced double-strand breaks	> 1	3
Unrejoined or misrejoined double-strand breaks (yeast)	> 1	1.3

For the assessment of the critical DNA lesion(s) leading to radiation-induced cellular transformation, the comparison of RBE- and OER-values for both endpoints may be useful. Table 1 shows that on the basis of their RBE- and OER-values, single-strand breaks, base damage and DNA-protein crosslinks are unlikely to play an important role in cell transformation. RBE- and OER-values of bulky lesions (accumulation of base damage at the nm-scale) have not yet been determined. However, it is unlikely that the RBE of these lesions increases with LET, since high LET radiations produce such high yields of DNA radicals in a nm-scale that a DSB rather than a bulky lesion may be produced.

The measurements of DSB were performed using the eukaryotic, unicellular yeast cell. The reason for this was that unrejoined or misrejoined DSB cannot yet be measured in mammalian cells at doses lower than 10 Gy (equivalent to about 400 initial DSB/cell). Figure 3 shows the non-linear relationship between unrejoined or

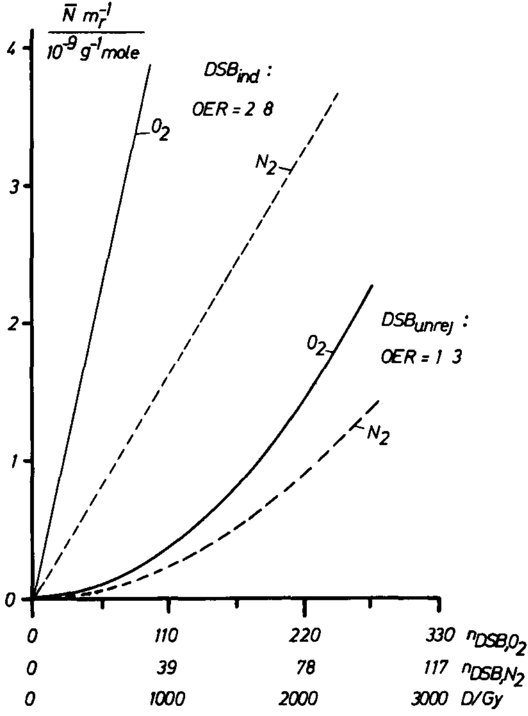

Fig. 3: The mean number $\bar{N}$ of initial and unrejoined or misrejoined DNA double-strand breaks in yeast per relative molecular mass m_r, $\bar{N}\, m_r^{-1}$, as a function of absorbed dose D. The irradiations were performed under aerobic or anoxic conditions. The lines represent the best fits to the experimental data as obtained by regression analyses.

misrejoined DSB and dose as well as the linear relationship between initial DSB and dose after irradiation under aerobic and anoxic conditions. The OER is 2.8 for initial DSB. This result is in agreement with those obtained in mammalian cells (17,18,19). In contrast, a low OER of 1.3 for unrejoined or misrejoined DSB was found.
DSB are the only DNA lesions exhibiting similar RBE and OER-dependencies (table 1) as cellular transformation. At high doses the OER of 2.9 observed for cell transformation is comparable to the OER of 2.8 for induced DSB. At low doses the OER is 1.5 for cell transformation which may be related to the OER of 1.3 observed for unrejoined or misrejoined DSB.
In conclusion, the comparison between RBE-values of densely ionizing radiations and of OER-values of sparsely ionizing radiations for the different DNA lesions and for cell transformation supports strongly the involvement of DSB in cell transformation.

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V. Publications:

1. Publications in Scientific Journals:

Frankenberg-Schwager, M., Frankenberg, D., and Harbich, R., Do DNA double-strand breaks lead to radiation-induced cell transformation? Abstract J.Cell.Biochem. Suppl. 14 A, 80, (1990).

2. Short Communications:

Frankenberg, D., The radiation-induced DNA double-strand break: Its induction and cellular consequences. Radiobiological Seminar, Neuherberg, 27. Nov. 1987.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-089-UK

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Title of the research contract:

The dosimetry and metabolism of incorporated radionuclides.

List of projects:

1. The dosimetry of inhaled radionuclides.
2. Metabolism and dosimetry of radionuclides in bone.
3. Mechanical transport of particles from the respiratory tract.
4. Translocation of material from particles deposited in the respiratory tract.

Title of the project no.: 1

The dosimetry of inhaled radionuclides

Head(s) of project:

Dr. G.N. Stradling

Scientific staff:

Miss S.A. Gray	Ms. C.G. Collier
Mr. J.C. Moody	Ms. M. Ellender
Mr. A. Hodgson	Miss M. Pearce

I. Objectives of the project:

To examine in rodents the behaviour of actinides that could be inhaled by humans as a result of occupational exposure or after their release into the environment. To undertake studies designed to understand the mechanisms involved in the translocation and clearance of radionuclides from the various regions of the respiratory system. To provide an experimental basis for assessing intakes of the actinides. To optimise treatment regimes for enhancing the removal of the actinides from the body after accidental intake.

II. Objectives for the reporting period:

1. To study the biokinetics of plutonium (Pu), americium (Am) and associated radionuclides in rodents after the alveolar deposition of residues formed at nuclear facilities.

2. To examine the biokinetics of uranium compounds formed in the fabrication of nuclear fuels.

3. To investigate the efficacy of DTPA, LICAM(C) and other chelating agents on the decorporation of actinides present in different chemical forms.

III. Progress achieved:

Inhalation facility

For administering aerosols of high toxicity eg. alpha emitters to small laboratory animals, a new inhalation facility has been built and commissioned. It comprises a suite of interconnecting gloveboxes which accommodates all the operations involved in an inhalation exposure. The exposure chamber has been designed to accept animal holding tubes of various sizes so that any combination of 36 animals ranging in size from mice to guinea pigs can be exposed simultaneously. In practice the main experimental animal is the rat and two of the exposure chambers are often connected in parallel. The operating characteristics of the exposure system were measured with monodisperse polystyrene and fused clay particles labelled with ^{99m}Tc and ^{111}In respectively. For particles with a mass median diameter (MMD) of 1.6 μm (σg 1.14), the coefficient of variation in the aerosol concentration at the exposure points was 10% with no positional bias. With larger particles (3.3 μm, σg 1.09) the coefficient of variation was 5%. Typically the initial lung deposit in rats exposed to the aerosols was 0.05% to 0.07% of the amount dispensed into the aerosol generator and for monodisperse particles with an MMD of 2 μm, the coefficient of variation between the animals was about 20%.

The new inhalation facility has been extensively used in the NRPB's expanding research programme on the biokinetics of actinide bearing dusts and the testing of chelating agents designed to enhance the elimination of transportable forms of actinides from the body. After the inhalation of plutonium dioxide, either alone or in combination with other metal oxides, other plutonium bearing residues and uranium oxides (MMD 1-2 μm), the coefficient of variation was in the range 10% to 22%. Lower values, about 8% were observed for plutonium nitrate vapour.

When information is required on the relationship between the systemic content of the actinides and their cumulative urinary excretion, or when insufficient material is available for inhalation experiments, the actinide bearing dusts are administered by intratracheal instillation.

Inhalation studies

Much of the published work on the biokinetics of the actinides after inhalation has involved studies with pure compounds eg. nitrates and oxides, often produced under laboratory conditions. Work conducted at the NRPB has demonstrated that it is not possible to use these data to assess the biokinetics of actinides present in complex mixtures of industrial

dusts. In the past five years studies have been undertaken with plutonium dioxide, either alone or in the presence of other oxides; residues formed in process lines or during the electrorefining of plutonium metal; actinide bearing dusts associated with the pond storage of spent nuclear fuel; dusts obtained from former nuclear weapons test sites and uranium compounds which are important intermediates in the nuclear fuel production cycle in the UK.

The aims of this work were to provide an experimental basis for recommending site specific Annual Limits on Intake (ALIs) and guidance for the interpretation of chest monitoring and urine analysis data. The biokinetics of the actinides in humans were predicted by combining the transfer rates to blood calculated from the animal experiments with mechanical clearance data obtained from human volunteers who had inhaled fused clay particles labelled with ^{85}Sr and ^{88}Y. This procedure is compatible with that likely to be adopted by an ICRP Task Group which is in the process of developing a new dosimetric model for the respiratory tract.

Plutonium-239 and americium-241 bearing materials

Experimental data obtained on the biokinetics of the ^{239}Pu bearing materials in rats indicated that whilst the ALI's ranged between those recommended by the International Commission on Radiological Protection (ICRP) for Class W and Y compounds, the rates of transfer of ^{239}Pu and ^{241}Am to blood (and hence the doses and risks to liver and skeleton) varied by more than two orders of magnitude (Table 1).

In general, the transfer rates for ^{241}Am are about 50% greater than for ^{239}Pu. However, in these materials it should be stressed that ^{241}Am is present as a daughter product of ^{241}Am and much greater discrepancies would be expected to occur if a mixture of both oxides were inhaled. The large difference in the transfer rate of the actinides after the inhalation of the residues obtained from the electrorefining process can be attributed to different chemical forms of the actinides. In such residues ^{241}Am is known to be present as the chloride whilst ^{239}Pu can be present as finely divided metal or an insoluble platinum complex, the so called black salt.

Table 1. Transfer rates of ^{239}Pu and ^{241}Am from the lungs of rats to blood and the values for Class W and Y compounds derived from the ICRP model for man.

Material	Actinide	Rates of transfer (% d^{-1}) 0-28d	28-365d
$^{239}PuO_2$	Pu	0.007	0.007
$^{239}PuO_2$	Pu Am	0.003 0.005	0.002 0.003
^{239}Pu + ^{235}U + Be oxides	Pu Am	0.011 0.012	0.005 0.008
$^{239}PuO_2$ + BeO	Pu Am	0.012 0.015	0.004 0.007
Nitrate bearing residues	Pu	0.33	0.050
Residues from refining process	Pu Am	0.071 1.46	0.009 0.041
Nevada test site material	Pu Am	0.31 0.41	0.13 0.18
Cooling pond residues	Pu Am	0.21 0.26	0.12 0.18
^{238}Pu + ^{241}Am nitrates	Pu Am	1.10 1.42	0.17[a] 0.22[a]
Class W compound		1.5	0.45
Class Y compound		0.067	0.016

a: 28-252d

The assessment of intakes of ^{239}Pu from measurements of ^{241}Am in the chest will depend on the transportability of the actinides, the particle size of the aerosol, the ratio of the actinides in the aerosol, the ALI and the limit of detection of ^{241}Am. In general, the particle sizes of the aerosols produced at the workplace were about 5 μm AMAD. Thus for class Y compounds eg. plutonium oxides and those with an appreciable class Y component, eg. nitrate bearing residues, the proposed ALI's are twice those recommended by ICRP for aerosols of 1 μm AMAD. For the materials cited in Table 1, the predicted biokinetics of the actinides in humans suggest that acute intakes of the least transportable forms of ^{239}Pu equivalent to the ALI could be variously assessed by this method from several months up to a

few years after exposure. On the other hand intakes of ^{239}Pu as nitrate equivalent to the ALI could be ascertained for up to a few days. Aerosols formed in the vicinity of cooling ponds contain a mixture of actinides and fission products. The experimental data suggested that measurements of ^{144}Ce or ^{137}Cs in the body is likely to be more advantageous than those of ^{241}Am for assessing intakes of ^{239}Pu.

The animal experiments showed that, for all the dusts investigated, the relationship between the systemic content (predominantly the liver and skeleton) and the cumulative excretion of ^{239}Pu was similar to that obtained after administration of the citrate. This indicated that the behaviour of ^{239}Pu in the body after its entry into the blood was independent of the chemical form of intake and that the interpretation of urinary excretion data based on models derived from human data after ^{239}Pu citrate administration would be valid.

The biokinetics of two of the materials cited in Table 1, namely $^{239}PuO_2$ and the Nevada test site material, are also being investigated in guinea pigs over a 4 yr period. After two years, the ^{239}Pu to ^{241}Am ratio in the lungs for the first material remained essentially unchanged, whilst for the second, it had increased by about 1.5 fold. This study will be used to provide guidance on the interpretation of chest monitoring data in the long term.

Uranium compounds

Uranium ore concentrates (UOC), trioxide (UO_3) dioxide (UO_2) and tetrafluoride (UF_4) are important intermediates in the production of nuclear fuels; UOC can consist of UO_3 or U_3O_8 or a mixture of both compounds.

The calculated transfer rates of uranium to blood after inhalation of these materials are shown in Table 2.

After the inhalation of UO_3, the rates of transfer of uranium to blood indicate that it should be assigned to inhalation Class D and not W as recommended by ICRP. Intakes should be restricted on the basis of chemical toxicity to a maximum of 2.5 mg d^{-1} and not on an annual limit of 5×10^4 Bq (2g of natural uranium) derived from dosimetric considerations.

After the inhalation of UF_4, the long term transfer rate of uranium to the blood suggests that the compound should be assigned to inhalation Class W for which the recommended ALI is 3×10^4 Bq. However since about 40% of the initial lung deposit of uranium translocates to the blood by 7d after exposure, then a limit on intake based on chemical toxicity would appear to be more appropriate.

Table 2. Transfer rates of uranium from the lungs of rats to blood, and the values for Class W and Y compounds derived from the ICRP model for man.

Material	Rates of transfer (% d^{-1}) 0-7d	7-84d
UO_3	32	2.3
UF_4	9.9	0.62[a]
U_3O_8	0.58	0.17
UO_2	0.25	0.061
UO_2[b]	0.18	0.033
Class W compound	4.7	0.51
Class Y compound	0.24	0.016

a: 7-350d b: ceramic form

After the inhalation of U_3O_8 and UO_2 the transfer rates to blood lie between those predicted for Class W and Y compounds. The long-term retention half-times of uranium in human lung calculated for U_3O_8, non-ceramic UO_2 and ceramic UO_2 using the procedure described previously are respectively about 230d, 430d and 520d. These values are compatible with those obtained from chest measurements of workers accidentally exposed to the same materials and lend support to the procedure used by NRPB for extrapolating the results of animal experiments to humans. The proposed ALI's for exposure to U_3O_8 and UO_2, respectively 10^4Bq and 5 x 10^3Bq, based on the particle size of the aerosol at the workplace (AMAD 6 μm) and the biokinetics of the uranium are about 6 times and 3 times the values recommended by ICRP for a 1 μm aerosol. The predicted behaviour of U_3O_8 and UO_2 in workers suggests that acute intakes corresponding to the ALI could be ascertained by chest monitoring up to more than 1y after acute exposure and that chronic intakes equivalent to 1/365 ALI per day could be detected with a 6-monthly monitoring interval or less.

Experiments have been undertaken to investigate the clearance of soluble forms of uranium from the three anatomical regions of the respiratory tract defined in the ICRP Lung Model. Small volumes, 2-3 μl, of uranyl nitrate and bicarbonate solutions were instilled into the nasal passage (N-P), trachea and bronchial tree (T-B) and pulmonary region (P) of the lungs of rats. The amounts of uranium which translocated to the blood by 10 days were, for both compounds, about 8%, 40% and 70% respectively of

the initial deposits. Otherwise most of the uranium was excreted in the faeces; only about 3%, 1% and 7% respectively was retained at the injection sites. The values obtained for the translocation of uranium to blood from the N-P and T-B regions are at variance with those postulated for the model for class D compounds, namely 50% and 95%, whilst the value postulated for the P region (100%) is in reasonable agreement with the experimental data. The results of this study suggest that for workers exposed to uranyl nitrate aerosols, the amounts of uranium that will translocate to the blood will be less than predicted by the model, 23% v 42% for particles of AMAD 1 μm. The likelihood of chemical damage to the kidneys will also be less than would be predicted for the model. In some industrial situations, class D uranium compounds such as uranium trioxide, are likely to be present as a dust with an AMAD of about 5 μm. Under these conditions even less uranium would be expected to be translocated to the blood because deposition will occur predominantly in the N-P region.

Thorium compounds

Occupational exposure to thorium is becoming of increasing concern due to the low values recommended by ICRP for the ALI. Currently, experiments are in progress to evaluate the effect of mass on the biokinetics of thorium inhaled as the nitrate. When the mass concentration of thorium in the lungs of rats is equivalent to about twice the value calculated to be in human lungs after an intake of ^{232}Th, about 10% of the initial deposit transfers to blood within the first few days after exposure. Thereafter, the transfer rate to blood is similar to that predicted for a class Y compound. When the mass concentration of thorium is reduced 10^6 fold, the amount transferred to the blood increases by only about a factor 2. Future work will examine the transportability of other industrial thorium compounds, eg. tetrafluoride, thoriated tungsten and dusts formed from mineral sands, and the relative biokinetic behaviour of thorium and its decay products since ^{228}Ac, ^{212}Pb and ^{208}Tl are used to assess the body content of ^{232}Th by whole body monitoring.

Enhanced excretion

In the event of accidental intakes of transportable forms of ^{239}Pu and ^{241}Am by workers, the only widely accepted method of treatment is the administration of the chelating agent diethylenetriaminepenta-acetic acid (DTPA). The calcium salt is recommended for prompt treatment and the zinc salt for protracted treatment due to its lower toxicity. Experiments

conducted at NRPB are concerned mainly with intakes of plutonium in which the mass concentrations in the lungs of rats are equivalent to those in human lungs after acute exposure to about twenty times the ALI. Under these conditions the repeated injection of DTPA (30 μmol kg^{-1} administered ip at 0.02, 0.25, 1.2 and 3d and then twice weekly from 6d to 24d) commencing 30 minutes after inhalation of plutonium as nitrate reduced the lung and total body contents by 28 days after exposure to about 1% and 2% respectively of those present in untreated animals. Similar results were obtained for americium inhaled as the nitrate. These data contrast strongly with those obtained in other laboratories in which DTPA had little effect on the decorporation of plutonium from the lungs when administered in concentrations equivalent to about 10^5 times the ALI for humans.

Since in mammals, Pu(IV) becomes associated with the Fe(III) transport and storage systems future developments in decorporation are likely to involve the use of synthetic analogues of siderophores; the latter are sequestering agents produced by micro-organisms in order to obtain Fe(III) from their enviroment. One such compound, a linear catechoyl amide code named LICAM(C), was reported in the literature as being at least as effective as DTPA for removing ^{238}Pu from animals after the intravenous injection as the citrate. This observation was subsequently confirmed in European laboratories which collaborated under the auspices of the European Late Effects Project (EULEP). However a likely route of accidental intake of the actinides under industrial conditions is by inhalation. Experiments conducted at NRPB showed that LICAM(C) was found to be substantially inferior to DTPA when administered after the inhalation of plutonium nitrate irrespective of whether it was administered as a polymeric, esterified or pure form. Using the same treatment regimen as above, pure LICAM(C) reduced the ^{238}Pu content of the lungs and total body by 28d to 61% and 58% of those in untreated animals. The other preparations of LICAM were even less effective.

The efficacy of two of the most promising synthetic analogues of siderophores, namely a hydroxypyridonate derivative of desferrioxamine (DFO-HOPO) and a dihydroxamic acid derivative of DTPA (ZnNa-DTPA-DX), have also been compared with DTPA. In these studies, $CaNa_3DTPA$ was used for the initial injection and $ZnNa_3DTPA$ at all other times. After intravenous injection of ^{238}Pu as citrate and the repeated intraperitoneal injection of the compounds (30 μmol kg^{-1} body wt at 0.02, 0.25, 1, 2 and 3d), the body contents of ^{238}Pu by 7d after exposure were reduced to respectively 8%, 29% and 15% of those in untreated animals. When the compounds were

administered at 0.02d only, the body contents were all about 50% greater than with repeated treatment. After the inhalation of ^{238}Pu as nitrate, the body contents of ^{238}Pu were reduced to 31%, 15% and 10% respectively of control values after repeated treatment when using the protocol above. As expected, DFO-HOPO was ineffective for enhancing the elimination of ^{241}Am after both methods of administration. However, after inhalation of ^{241}Am as nitrate, ZnNa-DTPA-DX was as effective as DTPA, the body content by 7d with repeated treatment being about 7% of control values. The results of these studies confirm the potential usefulness of synthetic analogues of siderophores for treatment of overexposure to the actinides. However at present, DTPA remains the chelating agent of choice.

The inhalation of thorium nitrate under industrial conditions is becoming of increasing concern due to the low values recommended for the Annual Limit on Intake (ALI) by ICRP. Yet, there is a paucity of data on the efficacy of treatment after this mode of intake. The efficacy of CaDTPA and ZnDTPA, the compounds of choice for other actinide elements, have been evaluated after the pulmonary deposition of thorium in widely different amounts. In the first experiment, the amount of thorium deposited (6.5 μg as $^{230+232}$Th) was similar to the mass concentration calculated to be in human lungs after acute exposure to twice the ALI for ^{232}Th. The prompt (300 or 1000 μmol kg^{-1} body wt at 0.02d) or repeated (30 or 300 μmol kg^{-1} at 0.02, 0.25, 1, 2, 3d) administration of CaDTPA were at best only moderately successful for enhancing the elimination of thorium by 7d after exposure. The body contents of $^{230+232}$Th at this time were respectively about 74%, 65%, 90% and 74% of those present in untreated animals. Under similar conditions of treatment ZnDTPA was less effective than CaDTPA. In the second experiment, thorium was administered as a carrier free form, ^{234}Th. However the substantial reduction in mass, about 10^7 fold, did not appreciably increase the efficacy of treatment. After the repeated administration of CaDTPA at doses of 30 and 300 μmol kg^{-1} using the protocol above, the body contents of ^{234}Th by 7d were respectively 69% and 51% of those in untreated animals. ZnDTPA was again less effective than CaDTPA.

These results differ markedly from those achieved for ^{238}Pu in which the body content was reduced to 10% of control values with CaDTPA by 7d with repeated doses of 30 μmol kg^{-1}. The development of more effective chelating agents for the decorporation of thorium are considered to be an important consideration for radiological protection of workers.

There appears to be no effective treatment for substantially enhancing the elimination of soluble uranium compounds from the body. Recently, it has been suggested that the administration of the sodium salt of 4,5-dihydroxy-1,3 benzene-disulphonic acid (Tiron) could be used as an antidote for uranium poisoning. We have investigated the efficacy of this substance (30, 300 or 1000 μmol kg^{-1} injected intraperitoneally at 20, 60 and 180 min) after the intratracheal instillation of uranyl nitrate in amounts which correspond to about 20 times the permissible daily intake of uranium by workers (2.5 mg). The body contents of uranium by 5d after exposure were respectively 107 $\pm$ 6, 78 $\pm$ 4 and 65 $\pm$ 5% of those in untreated animals. These results indicate that the development of more effective chelating agents is an important consideration for workers potentially exposed to transportable uranium compounds.

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V. Publications:

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Title of the project no.: 2

Metabolism and Dosimetry of Radionuclides in Bone

Head(s) of project:

Dr. J.D. Harrison
(Previously Dr. R.D. Saunders, Dr. N.D. Priest)

Scientific staff:

Ms. M.E. Ellender
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I. Objectives of the project:

To improve our understanding of the behaviour of bone-seeking radionuclides in the skeleton.

To provide information that can be used to improve assessments of doses to radiation sensitive cells in the skeleton following intakes of bone-seeking radionuclides.

II. Objectives for the reporting period:

To continue with studies of the distribution of ^{239}Pu, ^{241}Am and ^{233}U in mice, their retention in individual bones of the skeleton, and micro-distribution within bone. To continue with studies of the induction of osteosarcoma by these radionuclides.

III. Progress achieved:

Studies have been undertaken of the skeletal uptake and distribution within bone of radium, thorium, uranium, plutonium and americium, mainly in rodents. The results have shown that while each element is initially deposited on bone surfaces, they differ considerably in their distribution on different bone surfaces and may therefore deliver substantially different doses to sensitive cells on bone surfaces and in the marrow. For plutonium, comparisons have been made between rats, mice and baboons, indicating that the behaviour of the actinide in rodent bone can be used to predict behaviour in primate bone. The distribution of radium in bone was studied using pigs because of the close similarity between pigs and humans in alkaline earth metabolism. The results confirmed the initial deposition of radium on bone surfaces rather than throughout bone mineral. A study is currently in progress to compare the toxicity of ^{239}Pu, ^{241}Am and ^{233}U in mice. The objective is to relate the differences in the distribution of dose within the skeleton, and the extent of irradiation of different cell types, with the observed incidence and distribution of osteosarcoma. A low incidence of leukaemias is also expected.

The initial gross distribution of the actinides and radium between individual bones of the skeleton has been measured at one day after administration in young adult rats. The skeletons were stripped of their surrounding soft tissues by Dermestid beetles (Dermestes maculatus). They were then disarticulated and analysed for their radionuclide content. The relative concentration (RC) in individual bone was calculated as:

$$RC = \frac{\text{\% initial activity in bone per g ash weight}}{\text{\% initial activity in skeleton per g skeleton ash weight}}$$

In general, each element deposited preferentially in the main body of the spine, limb girdles and ribs, with lowest RC values for the paw bones, head bones and caudal vertebrae. This is consistent with published information and is considered to reflect differences in the blood supply to different parts of the skeleton. In addition, in the case of the head bones, low RC values for each element may be attributed, at least in part, to the presence of teeth which add greatly to ash weight but little to bone mineral surface to which the elements bind.

The variations between bones in the radionuclide concentrations was greatest for plutonium. For example, the concentration of plutonium in the lumbar and sacral vertebrae (RC of 2) was about six times greater than in the paw bones and mandibles (RC of 0.3). In contrast, the degree of variability exhibited by radium, uranium and americium was much lower; the maximum variation being about a factor of two from an RC of 1.4 to 0.6. Thorium gave intermediate results.

Previous studies of the initial distribution of ^{239}Pu in rat bone have demonstrated the predominant deposition of this element on endosteal bone surfaces. Little plutonium was deposited on periosteal surfaces and on the surfaces of the cortical vascular canals or on the walls of the vascular cavities adjacent to the epiphyseal plate cartilages. The surfaces of the trabeculae which form a band across the deeper regions of the primary spongiosa of the metaphysis also concentrate little plutonium. In contrast, the surfaces of other bone trabeculae and of the calcified cartilage remnants immediately underlying the epiphyseal plate cartilage were heavily labelled with ^{239}Pu. Thorium, which is chemically similar to plutonium in the stability of the tetravalent form, has been shown to have a very similar distribution.

Baboon bones containing either ^{239}Pu or ^{241}Am have been analysed as part of a collaborative study with the CEA, Bruyeres-le-Chatel, France. The autoradiographs obtained show that the actinides become deposited with a similar pattern to that observed in rodents. This confirms the general validity of experiments using rodents for examining the factors influencing the deposition of radionuclides in the skeleton. However, while plutonium in rat bone appears to be uniformly deposited on growing, resting and resorbing surfaces, enhanced levels of plutonium deposition in regions of bone resorption were observed in baboon bones. The reason for this difference is unknown. It is considered, however, that since resorption sites are relatively rare in adult human bone, the pattern of distribution in humans is likely to be characterised by even endosteal deposition as in rats.

Both plutonium and americium, initially deposited on bone surfaces, showed evidence of subsequent redistribution by bone growth and turnover. There was a tendency for surface deposited radionuclides to become relocated to form a more uniform volume deposit throughout the mineralised bone matrix. However, in the vertebrae, bone turnover seemed to be greater in the peripheral regions of the bone. Consequently, deposits of plutonium on more central bone surfaces persisted longer and this observation may go some way towards explaining why the spinal column is a preferred site for bone cancer induction. At all skeletal sites, some of the plutonium removed from bone surfaces by bone resorbing cells was found to be present in bone marrow macrophages. More plutonium was found in the macrophages of young animals than in those of adults which may be related to their higher rate of bone turnover.

Data obtained on the distribution and retention of plutonium in the baboon and rodent skeleton have been used in conjunction with physiological data on the human skeleton to construct an age-dependent dosimetric model and make estimates of doses to sensitive cells in the human skeleton(3). Calculations of the doses from ^{239}Pu to the red bone marrow and cells on bone surfaces using this model suggest that the current ICRP Publication 30 model gives a reasonable estimate of the doses at all ages, provided growth of the skeleton is taken into account. For ^{239}Pu reaching the bloodstream, the calculated integrated dose to the bone marrow to age 70 years, derived using the age-dependent model, varied from 40 to 110 Gy Bq^{-1} whereas the ICRP model gave values in the range 100-120 Gy. The corresponding values for the integrated dose to cells on bone surfaces were also lower than those calculated using the ICRP model but differed by less than a factor of three.

For adult man, the dosimetric model predicts that the fraction of a plutonium intake that transfers to marrow macrophages increases gradually to reach about 2 - 3% of the total body content after about five years after the intake. Thereafter, the model predicts that the level of plutonium in the bone marrow will fall to about 1% after 50 years. Of the total marrow activity, about two-thirds is predicted to be present in haemopoietic marrow rather than fatty marrow. These predictions are consistent with limited available data from measurements on ex-radiation workers.

The distribution of radium-226 in pig bone at 4 days after administration has been studied in collaboration with the MRC Radiobiolgy Unit(1). This period was chosen because of the uncertainties which exist concerning the distribution of radium-224 (half-life 3.64 days). ICRP assume that ^{224}Ra is deposited on bone surfaces while it has also been

suggested that it should be regarded as entirely or partially distributed throughout bone mineral. This distinction is important because extensive toxicity data are available for the effect of ^{224}Ra in man and its correct interpretation requires a precise knowledge of its distribution relative to sensitive cells. Autoradiographs of pig bone showed that the majority of the activity was present on bone surfaces. All bone surface were labelled with ^{224}Ra including the endosteal and periosteal surfaces and the surfaces of the vascular canals within the cortical bone. Further studies using tetracycline as a marker for bone growth also showed that growing bone surfaces concentrated more radium than resting and resorbing surfaces. The thickness of the bone surface deposits of radium were measured in collaboration with the U.S. Argonne National Laboratory. The results showed that radium was present as a very thin deposit on many bone surfaces but with some burial at other sites. This observation was taken to suggest that radium deposits as a thin layer on quiescent bone surfaces and as a broader layer on growing bone surfaces.

The available data on the relative toxicity in rodents of the long-lived isotope ^{226}Ra and the short-lived ^{224}Ra indicate that the latter is about six times more effective in causing osteosarcoma. This difference in toxicity has been attributed to differences in the distribution of these isotopes. It has been suggested that ^{224}Ra is more toxic than ^{226}Ra because it decays on bone surfaces rather than within the volume of the bone mineral. However, in rodents many bone structures are small compared with the track length of the α particles and this explanation may be inadequate to explain the magnitude of the observed effect. Consequently, Monte Carlo calculations have been made to test the validity of the distribution-difference hypothesis[2]. The results indicate that, for bone structures the size of those in mice, less than half of the observed difference in toxicity can be explained by consideration of the distribution of these radionuclides with respect to bone surfaces. Instead, it is suggested that the greater irradiation of trabecular than of cortical bone that is a characteristic of ^{224}Ra is responsible for its enhanced toxicity.

The study of the comparative toxicity of ^{239}Pu, ^{241}Am and ^{233}U in mice can be considered as consisting of four parts: radiochemical measurements of the distribution of the nuclides between the skeleton and soft tissues at times up to 448 days after intraperitoneal injection as their citrate complexes; radiochemical measurements of the retention of the nuclides in individual bones of the skeleton over the same period; autoradiographic studies of the distribution of the nuclides within bone; and comparisons of osteosarcoma induction in groups of mice given intraperitoneal injections of ^{239}Pu, ^{241}Am and ^{233}U activity to deliver equivalent average skeletal doses. The first part of the study is complete; the other three parts are continuing (Contract support extended).

The measurements of the tissue retention of ^{239}Pu, ^{241}Am and ^{233}U up to 448 days after a single intraperitoneal injection of 40kBq of each nuclide showed the expected rapid excretion of uranium compared with that of plutonium and americium. Thus, whole-body retention after one week was 68% of the injected activity for ^{239}Pu, 62% for ^{241}Am and 7% for ^{233}U. By the end of the 448 days of the study, whole-body retention had fallen to 20%, 21% and 2.5%, respectively, of the injected activity. At this time, the proportion retained in the skeleton accounted for 86%, 94% and 99% of the total whole-body activity, respectively. Similar results were obtained for male and female mice. The greatest skeletal accumulation occurred at 28 days after injection of Pu and Am and 7 days after injection of U. The

results obtained were used to calculate average bone doses over the 448 day period. The values obtained were 0.52 Gy for ^{239}Pu, 0.45 Gy for ^{241}Am and 0.10 Gy for ^{233}U. These dose estimates were used to determine the administered amounts for the main toxicity study as outlined below[4].

Measurements are in progress to determine the retention of ^{239}Pu, ^{241}Am and ^{233}U in the individual bones of the mouse skeleton with time after injection. The results for ^{239}Pu are complete and show that, as observed previously for rats, the greatest concentrations were generally in the main body of the spine, limb girdles and ribs, and lowest concentrations in paw bones and caudal vertebrae. Thus, the RC values obtained one day after administration were 2.0, 2.2 and 2.0 for the thoracic vertebrae, lumbar vertebrae and sternum, respectively, and 0.46 and 0.31 for the caudal vertebrae and paws, respectively. There was a trend towards a more homogenous concentration distribution with time.

Studies of the distribution of ^{239}Pu, ^{241}Am and ^{233}U within mouse bone at different times after injection have been carried out by CR39 autoradiography of femora and lumbar vertebrae. These studies have shown that Pu is predominantly deposited on endosteal bone surfaces. During remodelling and redistribution, some burial of Pu occurs at the epiphyseal plates and in cortical bone, but activity appears to remain largely on endosteal surfaces. With time, increasing amounts accumulate in the macrophages of the bone marrow. Americium deposits evenly on all bone surfaces and also on the internal bone surfaces of vascular canals in the cortical bone. With time, some burial can be seen at the growth plate and small amounts of activity are accumulated in the marrow macrophages. Uranium deposits on endosteal and periosteal surfaces but distribution is uneven with concentrations of activity in some areas. Small amounts of diffuse activity are seen throughout the bone marrow and bone mineral.

Fission track autoradiographs of femora have also been prepared for sections containing ^{239}Pu and ^{233}U. These have the advantage of having a bone image and will be used to make quantitative estimates of the distribution of activity on different bone surfaces, in bone volume and in the marrow. Similar measurements of the distribution of ^{241}Am will be made by superimposing CR39 autoradiographs and bone images produced by neutron bombardment. Photographic emulsion autoradiographs of mandibular condyle and rib are also being prepared for each nuclide to facilitate the identification of differences between the elements in their distribution on growing, resting and resorbing surfaces and their proximity to different cell types.

To compare osteosarcoma induction by ^{239}Pu, ^{241}Am and ^{233}U, each nuclide has been administered to three groups of 50 - 100 animals at different levels of activity. For ^{239}Pu, the three groups were given 5, 15 and 25 kBq kg^{-1}. The corresponding amounts of ^{241}Am to give equivalent average skeletal doses were 5.8, 17.2 and 28.9 kBq kg-1 and of ^{233}U were 39.5, 117.6 and 197.3 kBq kg-1. In each case the nuclides were administered by intraperitoneal injection as the citrate complexes in nine equal aliquots over a three week period. Control animals (100) were given injections of inactive solution. The average skeletal doses to 600 days in the low, medium and high groups are estimated as 0.13 Gy, 0.37 Gy and 0.63 Gy, respectively. The expected osteosarcoma incidences for ^{239}Pu are 7.5%, 25% and 40% in the three groups. The available published information on ^{241}Am suggests that the incidence may be about a factor of three lower than for ^{239}Pu; there are no data available to allow the prediction of incidence in the ^{233}U groups.

All mice in the osteosarcoma study are examined daily. Moribund animals and those with developed tumours or paralysis are killed. All animals are being X-rayed at death to determine the presence of osteo-sarcomas. Tumour classification is being carried out by histological examination. Animals in an additional high level ^{239}Pu group are being X-rayed periodically to follow tumour progression. To date about 20% of the animals have died. Most of the deaths (about 80%) have been due to liver tumours which are endemic in the CBA/H strain mice. Five bone tumours have been observed to date[5].

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

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Dr. H Métivier, Laboratoire de Toxicologie Experimentale, CEA, Bruyeres-le-Chatel, France.

Dr. N.D. Priest, Harwell Laboratories, UK.

V. Publications:

1. Priest, N.D., Green, D., Humphreys, J.E.M. and Haines, J.W., 1986, Autoradiographic techniques for the study of the distribution of radium in rat bone. Strahlentherapie 80 (Suppl.), 99-103.

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Title of the project no.: 3

Mechanical transport of particles from the respiratory tract

Head(s) of project:

Dr. M.R.Bailey

Scientific staff:

Dr. A. Birchall, Dr. R.A. Bulman, Ms. C.G. Collier, Mr. N. Dodd, Miss M.D.Dorrian, Dr. G. Etherington, Ms. S. Gray, Mr. N. Green, Mr. A. Hodgson, Miss S.P. Stewart, Dr. J.R.H. Smith.

I. Objectives of the project:

The overall objective of the project is to improve the scientific basis of models used to relate intakes of radionuclides by inhalation to tissue doses and to environmental and bioassay measurements, by:

(i) Measuring the rate at which discrete particles are cleared from different regions of the human respiratory tract: nasal passage, bronchial tree and pulmonary region.

(ii) Testing the hypothesis that the rates at which discrete particles are cleared from the lungs to the gastro-intestinal tract are independent of particle composition.

(iii) Investigating mechanisms of particle clearance from the lungs, and factors affecting clearance, including inter-species differences.

II. Objectives for the reporting period:

Development of techniques for studying particle clearance from the human respiratory tract, especially the nasal passage, and commencement of the experiments. Study of the biokinetics in man of intravenously administered yttrium-88.

Investigation in rodents of factors affecting mechanical transport rates from the alveolar region: alpha-irradiation, broncho-alveolar lavage, exercise, gender, age, route of administration, and particle composition. Investigation of lobar distribution of particles.

Development of models to describe mechanical transport of particles from the respiratory tract of humans and laboratory animals.

III. Progress achieved:

1. Methodology

The general approach used was to administer by inhalation, inert, monodisperse, radiolabelled particles. Respiratory tract retention and clearance were followed by external gamma-ray counting and excretion measurements, complimented in animal studies by serial sacrifices to determine tissue distribution. To obtain clearance rates due to mechanical transport of particles, correction must be made for particle dissolution, which may in turn require experiments to determine the biokinetics of the label. Considerable effort was put into developing techniques for producing test particles, and for administering them to human volunteers under controlled conditions.

Approval was obtained for a human volunteer study to determine the clearance kinetics of particles deposited in the nasal passage[2]. Subjects will inhale monodisperse polystyrene or fused aluminosilicate particles (FAP) labelled with ^{97}Ru ($t_{½}$ 2.8 d), or in pilot experiments, ^{99m}Tc ($t_{½}$ 6 hours). Equipment is being set up for separating ^{97}Ru from cyclotron-irradiated molybdenum powder by steam distillation. Following examination of the relevant polymer chemistry, HPLC procedures were developed for purifying a ruthenium-binding lipophilic chelate to label polystyrene with ^{97}Ru. Techniques were developed to produce monodisperse polystyrene particles in the wide size-range (0.5 - 50 μm) needed for the nasal clearance study[5,13,19]. A commercial spinning-disc aerosol generator was modified to increase its maximum speed, enabling particles as small as 1 μm to be produced at 20% efficiency. Although particles up to 50 μm could be produced, the technique was found to be inconvenient beyond 15 μm, because the droplets are dispersed into a ring of ~1 m diameter, resulting in problems of enclosure, shielding and recovery of the particles. A Vibrating Orifice Aerosol Generator (VOAG) was subsequently obtained, since the droplets emerge as a jet, and so are dispersed over a much smaller volume. Initial trials confirmed that the VOAG can also achieve greater uniformity, and higher output efficiency.

A laser aerosol photometer/spirometer, designed specifically for measurements of particle deposition in the respiratory tract, was obtained from GSF Frankfurt. This instrument, with other components of the aerosol administration apparatus, is operated under computer control. Software was written to enable rapid, simultaneous measurements to be made of the flow rate and aerosol concentration in inhaled and exhaled air. A bicycle

ergometer was installed to permit inhalations to be carried out at breathing patterns determined by set rates of working. Considerable inter- and intra-subject variation in nasal deposition and clearance is expected, partly due to morphological differences. Equipment to measure the cross-sectional area of the nasal passage as a function of distance into the nose by deconvoluting a reflected sound pulse was obtained from Aarhus University. Calibration tests and pilot studies were conducted, including measurements on casts of human nasal passages (adult, 6-year old and 1-year old). A detector system was set up to enable measurements of activity in the head, lungs and stomach to be made during and immediately after administration of labelled aerosols. Detector support stands were constructed and five 120 x 100 mm NaI(Tl) detectors installed. A micro-computer based multi-channel analyser (MCA) was obtained to record spectra.

Approval was obtained to study the biokinetics of ^{88}Y in man[17,18]. ^{88}Y ($t_{½}$ 107 d) is used as a label for FAP in long-term lung clearance studies, and the contribution to clearance made by particle dissolution is determined from measurements of its urinary excretion[3]. However, no information on the retention and excretion of tracer level yttrium in man following systemic uptake was known. ^{88}Y was administered by intravenous injection of 0.5 ml of 0.9% sodium chloride solution to which ^{88}Y citrate at pH 7 had been added. Following a pilot study in which one subject received 0.4 kBq, two subjects each received 4.0 kBq ^{88}Y. Total body and organ retention were measured at selected times up to one year. Quantitative faecal and urinary collections were made for 5 and 14 days respectively for one subject and for 14 and 24 days for the second. Subsequently, 72-hour urine samples were obtained at approximately monthly intervals. For comparison, groups of 4 rats were intravenously injected with the same ^{88}Y solutions.

In collaboration with Dr. Stahlhofen (GSF Frankfurt), FAP was prepared in which gold colloid labelled with ^{198}Au ($t_{½}$ 3 days) was incorporated, to obtain an exceptionally inert material (Au-FAP). It was also established that FAP is easily labelled with ^{111}In ($t_{½}$ 3 days), which is readily available and gives a lower dose per unit intake than ^{198}Au[13]. Assistance was provided in setting up a system at Frankfurt to generate monodisperse FAP. Dr. Stahlhofen's group have investigated human bronchial clearance by following retention of activity administered as a bolus of particles injected into the inspired air. When the particles were administered at the end of the breath, in order to deposit them primarily in the conducting airways, the fraction (F_s) which was not cleared rapidly

by mucociliary action was surprisingly large (~50%). Assistance was provided in one study, in which 6 subjects inhaled 3 μm aerodynamic diameter (AD) ^{198}Au-FAP[7,8]. One subject inhaled the particles under the same conditions as those under which he had previously inhaled 3 μm AD iron oxide particles. The others inhaled the bolus in a horizontal position, which was expected to result in more proximal deposition than when sitting upright.

Several animal studies on factors affecting particle clearance from the alveolar region to the gastro-intestinal (GI) tract (begun under the previous contract, BIO-D-489-UK) were completed. Particle retention and clearance were followed from 7 days to at least 6 months after intake. Retention of FAP was compared with that of a mixed actinide oxide, and the effects of alpha-irradiation, exercise, gender and route of administration were studied in rats[10,11]. The effect of broncho-alveolar lavage (BAL) on alveolar retention of FAP was studied in hamsters. Alveolar particle clearance rates were also estimated in the studies of the lung clearance of cobalt oxide particles described in detail under Project 4[6,9,14-16,20]. This enabled comparisons to be made of mechanical clearance rates of materials with different dissolution rates, and determination of the effect of age on mechanical transport.

A model to describe mechanical transport of particles from the human respiratory tract, suitable for predicting clearance kinetics and hence doses from inhaled radionuclides, is being developed in collaboration with other members of the ICRP Task Group on respiratory tract models[21]. Complimentary mechanical transport models for laboratory animals are being developed to determine translocation rates of materials from experimental data.

2. Results

(i) Particle clearance from the human respiratory tract.

The results of the pilot study on the biokinetics of ^{88}Y were consistent with the more accurate results obtained using the full activity, and good agreement was found between the results for the two subjects[17,18]. Total body retention (R(t) at t days after injection) in both cases was well characterised by a two-component exponential function:

$$R(t)/R(0) = 0.22 \exp(-\ln(2)t/0.65) + 0.78 \exp(-\ln(2)t/920)$$

The fraction of the total ^{88}Y excreted which appeared in urine (F_u) during the first 5 days was 0.94 and 0.93 for subjects A and B respectively. Measurements of the distribution of activity in the body were consistent

with most being deposited on bone surfaces. About 10% of the injected activity went to liver, of which half cleared rapidly ($t_{½}$ 25 d and 6 d in A and B respectively), and the rest with a half-life long compared to the time scale of the experiment. Results for rats were broadly similar to those for humans: ~25% of the injected activity was excreted in urine, mainly on the first day, and most of the retained activity was in the skeleton. However, faecal excretion was higher (F_u = 0.75), and liver retention lower than in man. The results for humans were generally in agreement with the ICRP 30 model for yttrium, which is based on animal data. Similarly there was no need to re-assess the contribution to clearance made by dissolution in the previous human lung retention study which used ^{88}Y-FAP[3].

The results of the study in which lung retention of 1 μm ^{85}Sr-FAP and 4 μm ^{88}Y-FAP were followed in humans for up to a year[3] were, however, re-analysed[12]. Since it was considered possible that using two-component exponential functions to represent lung retention might have influenced the estimated clearance rates, in particular resulting in relatively constant rates beyond 150 d, polynomial functions were fitted to the original data. The new analysis confirmed that the clearance rate from the lungs to the GI tract decreases from ~0.3% of the remaining lung content per day at 25 d after inhalation, to ~0.1% per day at 150 d, but indicated that it continues to decrease, to 0.05% per day at 350 d. Intersubject variation was also characterised. At 200 d after inhalation, most of the results conformed to a log-normal distribution with a median of 0.08% per day, and geometric standard deviation of 1.6.

In the investigation of bronchial clearance conducted in collaboration with Dr. Stahlhofen at Frankfurt[7,8] it was found that for the seated subject the slow-clearing fraction of the lung deposit F_s, was ~50%, as found before with iron oxide particles, confirming that the result is independent of the test material. F_s was even higher, about 75%, for the subjects who inhaled the bolus in a horizontal position.

(ii) Animal experiments.

Lung retention was followed for up to 640 d in rats that inhaled a mixed actinide oxide containing ^{239}Pu and/or ^{57}Co-FAP[10]. Lung retention of ^{239}Pu in rats exposed at the lowest level (initial lung deposit 0.1 kBq α-activity) was greater than that of ^{57}Co, the difference being attributed to slower dissolution of the FAP. At the higher levels of actinide (2 and 9 kBq), greater retention of ^{239}Pu occurred, and at the highest level,

greater retention of FAP also, these effects being attributed to impaired clearance resulting from α-radiation induced lung damage. Exercise produced no significant effect on alveolar clearance of ^{57}Co-FAP, nor was there a difference between males and females[11]. The clearance of intratracheally instilled ^{57}Co-FAP was significantly faster than that of inhaled particles up to about 30 d after intake, but not thereafter[11]. Bronchoalveolar lavage (BAL) at 7, 30 or 200 d after inhalation of ^{57}Co-FAP by hamsters removed 66%, 59% and 68% of the lung content at the start of treatment. The fractional clearance rate of the material remaining in the lung after BAL was similar to that in the control animals. The results suggest that BAL can be an effective treatment even if delayed and that it does not selectively remove material which would clear relatively rapidly.

Alveolar mechanical clearance rates, M(t), were estimated for ^{57}Co-labelled cobalt oxide particles ($^{57}Co_3O_4$) in the studies described in Project 4. For rats and hamsters that inhaled 0.8 μm and 1.7 μm "porous" $^{57}Co_3O_4$, values of M(t) for the two particle sizes were similar in each species, even though their dissolution rates were different[6,9,14,15]. They were also similar to values of M(t) in rats and hamsters respectively, obtained from previous experiments with FAP[4], which dissolves in the lung much more slowly than $^{57}Co_3O_4$. No significant difference was found between values of M(t) for rats which inhaled "dense" $^{57}Co_3O_4$, at the ages of 3, 13, 21 and 46 weeks[16,20]. In all groups the clearance rate decreased from ~3% of the remaining lung content per day at a week after inhalation, to ~0.5% per day at 9 months. Values of M(t) were similar to those observed in rats in the study using "porous" $^{57}Co_3O_4$, and in studies using FAP.

Progress was made on developing a model to describe mechanical transport of particles from the human respiratory tract. In the current version, time-dependent clearance rates are represented by combinations of first-order compartments to avoid problems with chronic exposures and radioactive decay products[21]. Interim estimates of representative values and ranges have been made. A model for rats has also been developed, using the same compartmental structure, but with appropriate values. The exercise has, however, highlighted the difficulties involved in estimating many of the parameters needed from available data.

3. Discussion

Since administrative problems delayed the planned measurements of particle clearance rates from the human respiratory tract, greater effort

was placed on animal studies, on which European collaboration opened up unexpected opportunities.

The facilities for preparing test particles and administering them to human volunteers were extensively upgraded. Although initially required to study particle clearance from the nasal passage, they will be applicable to other projected human inhalation studies, especially those requiring particle administration to a selected part of the respiratory tract. There remain major uncertainties about the kinetics of clearance of particles from each part of the human respiratory tract, even for healthy adult males[1,12]. Of particular current concern is the possibility, suggested by the Frankfurt experiments, that a significant fraction of material deposited in the TB region may not be cleared rapidly by mucociliary action, since the TB epithelium is regarded as being of relatively high radiosensitivity. These uncertainties were highlighted by work on the development of a model to represent mechanical clearance kinetics. It is planned to commence the nasal clearance study shortly, and further studies of human lung clearance in due course. Closer collaboration on human inhalation studies is foreseen with GSF Frankfurt and also with CEA Fontenay-aux-Roses, with whom a joint proposal to the CEC Radiation Protection Programme 1990-91 was submitted and selected for funding.

Studies in rodents provided further support for the assumption that mechanical transport rates from the respiratory tract are independent of particle composition. This is important for applying the results of human studies using non-toxic materials to the prediction of clearance of toxic materials. Similar rates of mechanical clearance from the alveolar region to the GI tract were found for FAP, an actinide oxide, and three different forms of $^{57}Co_3O_4$. The alveolar mechanical clearance rate was generally found to be remarkably constant in a given species. It is largely independent of age and gender, and unaffected by voluntary exercise, or even BAL. High levels of α-irradiation were found to impair it, and the rate was found to be higher (but only initially) for particles administered by instillation than by inhalation.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

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Dr. H. Toivonen
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V. Publications:

1. M.R. Bailey (1985). Human data on clearance. IN Lung Modelling for Inhalation of Radioactive Materials. Proceedings of a Meeting Jointly Organized by the CEC and NRPB, Oxford 26-28 March 1984 (Eds. H.Smith and G.B.Gerber) CEC pp.93-110.

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Title of the project no.: 4

Translocation of material from particles deposited in the respiratory tract.

Head(s) of project:

Dr M R Bailey

Scientific staff:

Miss C G Collier, Mr A Hodgson, Dr J W Stather, Mr J Moody, Miss S Gray, Mr A Ball

I. Objectives of the project:

To improve the scientific basis of models used to relate intakes of radionuclides by inhalation to tissue doses and to environmental and bioassay measurements, by:

(i) Interspecies comparison of translocation of materials from particles in the lung to the blood.

(ii) Measurement of absorption to blood of materials deposited in the anterior or posterior nasal passage of human subjects.

(iii) Measurement of absorption to blood and retention in the epithelium of materials deposited in the nasal passage of rodents.

II. Objectives for the reporting period:

(i) Design, construct and test new facilities for administration of radioactive aerosols to rodents.

(ii) Compare the rate of translocation to blood of ^{57}Co following inhalation of $^{57}Co_3O_4$ by rats, hamsters and guinea-pigs and by rats of different ages.

(iii) Investigate factors affecting translocation to blood of ^{57}Co from $^{57}Co_3O_4$ in the lungs, to account for interspecies differences: effect of medium on $^{57}Co_3O_4$ dissolution; measurement of intraphagolysosomal pH; comparison of transfer of soluble ^{57}Co from lungs to blood in different species.

III. Progress achieved:

<u>Methodology</u>

(i) Experimental studies on translocation to blood of material deposited in the lungs frequently require exposure of animals by inhalation. A new facility for administering radioactive aerosols to small animals was designed and constructed[3,8]. A suite of interconnecting gloveboxes was designed to accommodate all of the operations involved. A new exposure chamber was designed to reduce the variation between individual animals and permit exposure of animals ranging in size from mice to guinea-pigs. With two chambers in parallel, simultaneous exposure of up to 72 animals is possible. The new system was tested extensively using radio-labelled fused aluminosilicate and polystyrene particles (1-3.5 μm AMAD). Variation in lung deposit between individual rats was found to be 15-20% (compared to 40% with the previous system) and the efficiency (activity deposited in each animal's lungs/activity nebulised) was around 7×10^{-2}%. The system has been fully operational since 1987, and more than 12 exposures have been conducted, using materials such as thorium nitrate, plutonium nitrate, plutonium oxide and cobalt oxide.

(ii) An inter-laboratory collaborative study[1,2,4,5,7] was conducted primarily to test the hypothesis that translocation of materials from the lungs to the bloodstream is independent of the animal species. 'Porous' cobaltosic oxide labelled with ^{57}Co ($t_{½}$ 271 d) ($^{57}Co_3O_4$) was chosen as the test material as it is moderately soluble and most systemic cobalt is rapidly excreted in the urine. Thus translocation rates could be estimated from urinary excretion rates and a simple biokinetic model. Monodisperse $^{57}Co_3O_4$ particles (0.8 and 1.7 μm mean geometric diameter MGD) were prepared at GSF Neuherberg and distributed to the participating laboratories to be administered to a wide range of species: GSF (dogs), CEA Bruyères-le-Châtel (baboons), KFK Karlsruhe (rats), MRC Chilton (SPF rats), Harwell laboratory (mice), AEE Winfrith (Man) and NRPB (rats, hamsters and guinea-pigs).

Particles were administered to all experimental animals early in 1985. The human exposure however, did not take place until February 1986 and so a second group of rats were exposed at NRPB to establish whether storage had altered the characteristics of the material. Animals at NRPB were exposed by nose-only inhalation. Total retention, organ distribution and excretion of activity were measured for 400 days after exposure. Groups of animals were killed at specific times after exposure and the

activity in the lungs measured. This allowed the lung retention to be determined from the measurements of whole body activity made on living animals.

Translocation rates were calculated from urinary excretion. Allowance was made for ^{57}Co cleared by translocation which was excreted in the faeces, and also for the systemic uptake of ^{57}Co from particles cleared to the GI tract. This was done using a simple biokinetic model, the parameters for which were determined in supplementary experiments. The whole body retention and excretion of ^{57}Co was measured in animals of each species injected intravenously with ^{57}Co-nitrate, and in animals which had been fed $^{57}Co_3O_4$ particles. Measurements continued for up to 3 weeks after exposure. Translocation rates were estimated in a similar way for all species in the interlaboratory collaboration and the results for different species were compared.

(iii) For the study on the effect of animal age on lung clearance[6] kinetics a different batch of $^{57}Co_3O_4$ was used. This had a MGD of 1 μm and was denser than that used in the previous study. It was again supplied by Dr W.G. Kreyling. Groups of rats ranging in age from 3 weeks to 1 year were exposed simultaneously to an aerosol of the particles in the new inhalation facility. Following the same experimental procedure as for the interspecies comparison (above), the lung retention, translocation and mechanical clearance of ^{57}Co were followed for 9 months after exposure. Estimation of clearance rates from excretion rates required supplementary experiments on the retention and excretion of ^{57}Co following systemic uptake and the uptake of ^{57}Co from particles passing through the GI tract for animals of different ages. The translocation rates obtained for adult rats for this 'dense' $^{57}Co_3O_4$ were compared with those obtained previously for the 'porous' material and with translocation rates of the same 'dense' material measured in dogs and baboons which had been exposed to the same material. The results for these three species for the 'dense' $^{57}Co_3O_4$ formed a second interspecies comparison of translocation rates[9].

(iv) Results from the two interspecies comparisons[5,9] suggested species differences for translocation rates of the same material. Possible reasons for these differences were investigated. The effects of the composition of the medium on the in vitro dissolution of $^{57}Co_3O_4$ were investigated in a simple system. $^{57}Co_3O_4$ particles were suspended in solutions of selected concentrations of citrate, bicarbonate and pH. The suspensions, contained in dialysis tubing, were immersed in similar solutions from which samples were removed for analysis. Measurements continued for 3 months.

(v) Since the initial stage of translocation (dissolution of the particles) takes place mainly within macrophages, species differences in pH within the lysosomes were investigated. Cells were recovered by lavage from guinea-pigs and transported to the Karolinska Institute, Stockholm, Sweden. Cells from dogs, baboons and rabbits were provided by other laboratories. The cells were incubated with fluorescein-labelled silica particles. The pH within the phagolysosomal vacuole was determined by UV fluorescence spectrophotometry. Results were compared for the different species.

(vi) Another possible reason for the species differences in translocation rates was investigated. Following dissolution of ^{57}Co from the particles, material is transferred to the blood. Species differences in this pathway could result in differences in translocation rates even if dissolution of the particles occurred at the same rate. This possibility was investigated by comparing the retention of ^{57}Co following instillation of $^{57}CoCl_2$ solution into the lungs of rats and guinea-pigs. Information was already available for dogs and further studies are planned using baboons and mice. Whole body retention, excretion and tissue distribution of ^{57}Co was followed for 3 months after instillation of about 150 kBq of $^{57}CoCl_2$. In addition samples of trachea were taken for autoradiographic studies to determine the distribution of the activity within it.

Results and Discussion

(i) Interspecies comparison of translocation rates[1,2,4,5,7].

Lung retention and clearance were followed for 400 days after inhalation of $^{57}Co_3O_4$ particles by rats, hamsters and guinea-pigs. Lung retention and excretion of ^{57}Co was similar in rats exposed in 1985 and in 1986, indicating that storage of the material had not affected its lung clearance characteristics.

By 400 days after exposure to 0.8 μm $^{57}Co_3O_4$, 0.08, 0.37 and 0.1% of the initial lung content remained in the lungs of rats, hamsters and guinea-pigs respectively. For animals exposed to 1.7 μm $^{57}Co_3O_4$, 1.6, 1.65 and 1.27% respectively remained in the lungs.

The rate of translocation of ^{57}Co from the lungs to the blood (S(t) at time t after inhalation) was calculated as a fraction of the activity remaining in the lungs. In all three species studied at NRPB, S(t) could be determined from about 10 to 350 days after exposure. For the 1.7 μm particles initial values of S(t) were similar (0.3% d^{-1} in guinea-pigs to 0.6% d^{-1} in rats). In all three species it increased slowly with time to

about 1% d^{-1}, reaching this value at about 150, 250 and 300 days respectively for the guinea-pigs, rats and hamsters. For the 0.8 μm particles S(t) was generally higher than for the 1.7 μm particles, showed more pronounced changes with time and greater differences between species. Initial values of 0.7, 1.6 and 0.6% d^{-1} for guinea-pigs, rats and hamsters respectively, increased to a peak of about 3% d^{-1} at 350, 50 and 250 days respectively. In rats the value of S(t) fell markedly again to about 0.5% d^{-1} by the end of the experiment.

A simple model of translocation of ^{57}Co associated with particles in the lungs to blood was developed, which was well able to account for the different forms of S(t) observed, including those for the other species used in the interlaboratory collaboration. The form of the predicted translocation rate as a function of time depended upon two variables: the initial dissolution rate and the fraction of material dissolved from the particles which was retained in the lung with a long half-time.

(ii) Effect of animal age on lung clearance kinetics[6].

Deposition of $^{57}Co_3O_4$ in the lungs increased with age. Lung retention, expressed as a percentage of the lung content on day 7 after exposure, was significantly lower in the youngest animals (3 weeks at exposure) than in adults. Aged animals (46 weeks at exposure) showed significantly higher lung retention than did young adults. Translocation rates (S(t)) were estimated from the urinary and faecal excretion rates, corrected by the transfer coefficients determined in the supplementary metabolic experiments.

In all age groups S(t) tended to increase with time from about 1% d^{-1} to 1.5-4% d^{-1}. Analysis of variance showed that between groups of different ages there were no significant differences in the mechanical clearance rates, but there were significant differences in S(t) ($p<0.01$), with the youngest animals having the highest translocation rates (1.5-2 times higher). The results suggest that provided mature animals are used, the effect of age on translocation rates to blood is minimal. In extrapolation of translocation rates in animals to those in humans, the differences in the rates between the species (see above) are likely to be much larger than between animals of different ages. However, the influence of age should be considered when extrapolating from data obtained using very young animals or when attempting to predict the translocation rates in children.

Exposure of rats to 'dense' $^{57}Co_3O_4$ formed part of a second inter-laboratory collaborative study[9]. The lung retention and clearance of

this material was compared in rats, baboons and dogs and with the results for the 'porous' $^{57}Co_3O_4$ used previously. S(t) was about 3 times lower for the 'dense' particles than the 'porous' ones in all three species, supporting the assumption that S(t) is proportional to specific surface area, which was calculated to be about 3 times higher for the 'porous' than for the 'dense' $^{57}Co_3O_4$ particles of the same size. In all cases, the time dependence of the translocation rate could be predicted by the simple model described above.

(iii) Factors affecting in vitro dissolution of $^{57}Co_3O_4$.

Of the factors studied, pH had the most significant ($p<0.001$) effect on dissolution of $^{57}Co_3O_4$ in vitro. The highest dissolution of $^{57}Co_3O_4$ occurred at pH 4.5 (generally 2-6 times higher than at pH 7.2). Citrate concentration also had a significant effect on dissolution rates ($p<0.02$), with particles in 0.2 mM citrate dissolving slightly faster than in 0.067 mM citrate. Bicarbonate concentration had no effect on dissolution and there was no evidence of synergism between the factors. These results suggested that pH in the dissolving environment (alveolar macrophage lysosome) is likely to be a major factor in determining particle dissolution rates.

(iv) Measurement of intraphagolysosomal pH.

Measurements were conducted on alveolar macrophages from guinea-pig, rabbit, dog and baboon. For all four species the pH was found to be 4.5-4.6, with little variation between individuals. These species showed wide variation in translocation rates of ^{57}Co from $^{57}Co_3O_4$, and yet the pH in the lysosomes was very similar, indicating that another factor must be responsible for the differences.

(v) Comparison of retention of soluble cobalt in the lungs.

Retention of ^{57}Co was followed for 100 days after instillation, by which time total body retention was 0.93 and 1.4% of the administered activity in rats and guinea-pigs respectively. A two component exponential function fitted to the whole body retention data showed that for both species around 95% of the activity was cleared within the first day, and the remaining 3.5-5% was cleared with a 45 day half-time. Animals were killed at 7, 30 and 100 days and the tissue distribution of activity determined. At all times the lungs contained >25% of the whole body activity and the highest activity of any organ. For both species throughout the experiment the highest concentration of activity was in the trachea (100-600 times the average throughout the body). The lungs also showed high concentrations (45-150 times the average concentration).

Overall, the results for excretion and retention were similar for the rats and guinea-pigs and also similar to those reported previously for hamsters and beagles. All four species retained a similar fraction of dissolved activity (3-5%) in the lungs, and the range of values could not account for the differences observed between species in translocation rates.

Interestingly both rats and guinea-pigs showed preferential accumulation of ^{57}Co in the trachea. This activity was not associated with the administration site. Tissue samples from the experimental animals have been taken for autoradiographic examination. Initial results suggest that cobalt accumulates in the cartilage rings of the trachea.

Much of the work has been of a collaborative nature. This has required considerable effort in the co-ordination of the work and the presentation of the results for publication.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

One representative given for each laboratory (others and full addresses given in publications).

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Dr P. Camner, Karolinska Institutet, (KI), Stockholm, Sweden.
Dr H. Métivier, CEA, Bruyères-le-Châtel, France.
Dr E. Drosselmeyer, Karlsruhe, West Germany.
Dr G. Patrick, MRC Radiobiology Unit, Chilton, UK.
Dr A. Morgan, Harwell Laboratory, Oxon, UK.
Mr P. Foster, Atomic Energy Establishment Winfrith, UK.
Dr S. Pickering, CEC, Karlsruhe, West Germany.

V. Publications:

1. Bailey, M.R., Kreyling, W.G., Andre, S., Batchelor, A., Black, A., Collier, C.G., Drosselmeyer, E., Ferron, G.A., Foster, P., Haider, B., Hodgson, A., Métivier, H., Moores, S.R., Morgan, A., Muller, H.-L., Patrick, G., Pearman, I., Pickering, S., Ramsden, D, Stirling, C., and Talbot, R.J. (1986). An interspecies comparison of the lung clearance of inhaled monodisperse cobalt oxide particles. In: Proceedings of The Second International Symposium on Deposition and Clearance of Aerosols in the Human Respiratory Tract. Salzburg 18-26 Sept. 1986. (Edited by W. Hofmann) Facultas Universitatsverlag pp119-122.

2. Collier, C.G., Bailey, M.R., Hodgson, A. Lung clearance of cobalt after inhalation of 0.8 μm and 1.7 μm monodisperse cobalt oxide particles by hamsters, rats and guinea-pigs. EULEP Newsletter No. 42, June 1986.

3. Hodgson, A., Moody, J.C., Bailey, M.R., Stather, J.W. (1987). A new system for administering radioactive aerosols to rodents. In: Aerosols: Their Generation, Behaviour and Applications. Abstracts of 1st meeting of The Aerosol Society held at Loughborough University, 31st March - 1st April 1987.

4. Bailey, M.R., Kreyling, W.G., Andre, S.A., Batchelor, A., Black, A., Collier, C. G., Drosselmeyer, E., Ferron, G., Foster, P., Haider, B., Hodgson, A., Métivier, H., Moores, S.R., Morgan, A., Müller, H.L., Patrick, G., Pickering, S., Ramsden, D., Stirling, C., and Talbot R. (1988). An interspecies comparison of the translocation of material from lung to blood. Ann. Occup. Hyg. Supplement 1. Inhaled Particles VI. 975-986.

5. Bailey, M.R., Kreyling, W.G., Andre, S., Batchelor, A., Collier, C.G., Drosselmeyer, E., Ferron, G., Foster, P., Haider, B., Hodgson, A., Masse, R., Métivier, H., Morgan, A., Müller, H.-L., Patrick, G., Pearman, I., Pickering, S., Ramsden, D., Stirling, C. and Talbot, R.J. (1989). An interspecies comparison of the lung clearance of inhaled monodisperse cobalt oxide particles. J. Aerosol Sci. 20 No 2. 169-188.

6. Collier, C.G., Hodgson, A., Gray, S.A., Moody, J. and Ball, A. (1989). Study of the effect of age on lung clearance kinetics in rats. In: Radiation Protection-Theory and Practice. Presented at the 4th International Symposium of the SRP, Malvern June 1989.

7. Collier, C.G., Bailey, M.R. and Hodgson, A. (1989). An interspecies comparison of the lung clearance of inhaled monodisperse cobalt oxide particles- Part V : lung clearance of inhaled cobalt oxide in hamsters, rats and guinea-pigs. J. Aerosol Sci. 20 No 2. 233-248.

8. Hodgson, A., Moody, J.C., Bailey, M.R., Stather, J.W. and Stradling, G.N. (1989). A facility for administering actinide aerosols to rodents. NRPB M-171. National Radiological Protection Board, HMSO.

9. Kreyling, W.G., Andre, S., Collier, C.G., Ferron, G.A., Métivier, H. and Schumann, G. (1989). Interspecies comparison of lung clearance after inhalation of monodisperse, solid cobalt oxide particles. Proceedings of Joint European Aerosol Conference Vienna Sept. 1989. To be published in J. Aerosol Sci.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-196-I

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Title of the research contract:

Regulation of DNA methylation in cell differentiation, transformation and repair.

List of projects:

1. Regulation of DNA methylation in cell differentiation, transformation and repair.

Title of the project no.: B16 - D - 196 - I

Regulation of DNA methylation in cell differentiation, transformation and repair.

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(Dr. A. Reale)	(Dr. M. Rispoli)	(Dr. A. Tomassetti)

I. Objectives of the project:

Under the general biochemical assumption that hypomethylation of eukaryotic genes is correlated to their expression, and taking into account that gene demethylation can be expected to behave as a somatically inheritable event with mutation-like effects but occurring with a higher frequency than true mutation, it was proposed to investigate, using an in vitro cellular system capable of cell differentiation under controlled conditions, how the DNA methylation pattern correlates to the cultural and/or differentiation characteristics of the cells, and in particular to the phenotypic changes induced by sublethal exposure to ionizing radiation.

II. Objectives for the reporting period:

1) Effects of sublethal exposure to X-irradiation on specific gene expression by cultured cells endowed with a DNA-methylation-sensitive tendency to undergo terminal differentiation.
2) Effects of DNA methylation inhibitors on the susceptibility to radiation damage of cultured mammalian cells.
3) Evaluation of single-strand breaks occurring in nuclear DNA during cell differentiation and/or exposure to X-rays.
4) Identification of factors which regulate, within chromatin structure and at the single gene level, the susceptibility to in vitro methylation of particular CpG sequences in DNA.

III. Progress achieved:

1. Methodology.

The main cellular system used was the L5 rat myoblast line (or, rather, subclones derived from it). These cells can either be maintained in a proliferating, undifferentiated state, or can be induced to differentiate and fuse into multinucleated myofibers simply by decreasing the foetal calf serum (FCS) concentration in the cell culture medium (Scarpa et al., Cell Diff. 17, 105, 1985). Three clones were chosen -- M6, M9 and M12 -- which differ in their tendency to undergo spontaneous fusion or in their susceptibility (in terms of acceleration and/or facilitation of the differentiation process) to the presence, in the culture medium, of the adenosine analog 3-deazaadenosine (DZA) -- the effect of this drug being potentiated by the simultaneous presence of homocysteine (Scarpa et al., Proc.Natl.Acad.Sci.USA 81, 3064, 1984).

All myoblast clones were grown in F14 medium additioned with 10% FCS ("growing medium"), and eventually transferred to F14 medium with only 1% FCS ("fusion medium") in order to induce differentiation and fusion into myofibers. The extent of differentiation was evaluated by computing the number of nuclei found in fibers vs the total number of nuclei. The effects caused on the three clones by the addition of 3 μM DZA and 50 μM HCY can be described as follows: clone M6, normally fusing to a level of 50% when transferred to the "fusion medium", was stimulated to 75% fusion by addition of DZA+HCY; clone M12 failed to differentiate spontaneously but reached up to 50% fusion upon addition of the two drugs; clone M9 had the most abnormal behaviour, since at early passages (less than 5 transfers after cloning) its fusion was partially inhibited by the presence of the drugs, while after several passages (over 15 transfers after cloning) it exhibited a "typical" fusion pattern, very similar to that of M6. Every one of the three clones possessed a normal karyotype (42 chromosomes, as in normal rat tissues) and exhibited, upon X-irradiation, survival curves with a typical "shoulder" in the 0-5 Gy region, similar to those of other rodents cell lines such as CHO and V79.

In our experiments, the cells from a given clone were subjected to X-irradiation (the radiation dose being usually in the 0-5 Gy range) either in the "growing medium" or in the "fusion medium"; methylation inhibitors were, if the case, added after irradiation, and the effects on cell differentiation and survival were investigated. The occurrence of radiation-induced single-strand breaks of DNA was monitored, in cells which had been pre-labeled for 48 hours with radioactive thymidine, by the DNA alkaline elution technique (Kohn et al., Biochemistry 15, 4629, 1976) -- this procedure being also capable of detecting more complex structural differences in chromatin between differentiatied and undifferentiated cells. The 5-methylcytosine levels were measured by high

performance liquid chromatography in two different systems or, after suitable derivatization, by a gas chromatographic procedure coupled to mass spectrometry.

An independent study of the possible effects of methylation inhibitors on the susceptibility of cultured mammalian cells to radiation damage was also performed by investigating, in human lymphocytes and in chinese hamster ovary (CHO) cells, sister chromatid exchange, which was then correlated to the levels of cytosine methylation in the isolated DNA from these cells.

Standard chromatin fractionation procedures, previously developed in our laboratory, were used to investigate how chromatin structure -- in particular through its tightly-bound protein constituents -- can influence the susceptibility of its DNA to undergo in vitro methylation, and how the enzyme which catalyzes this process -- i.e. DNA-methyltransferase -- is distributed within the structural levels of eukaryotic chromatin. The possibility of an intrinsic resistance of some well-defined polydeoxyribonucleotide sequences to the action of the enzyme was also investigated, using as substrates either synthetic polydeoxyribonucleotides of known composition, or genomic clones obtained by inserting, in plasmid vectors, some naturally occurring "CpG-rich islands" (which are reportedly, in vivo, in a non-methylated state) or non-island-associated genes. A new assay for DNA-methyltransferase was also developed.

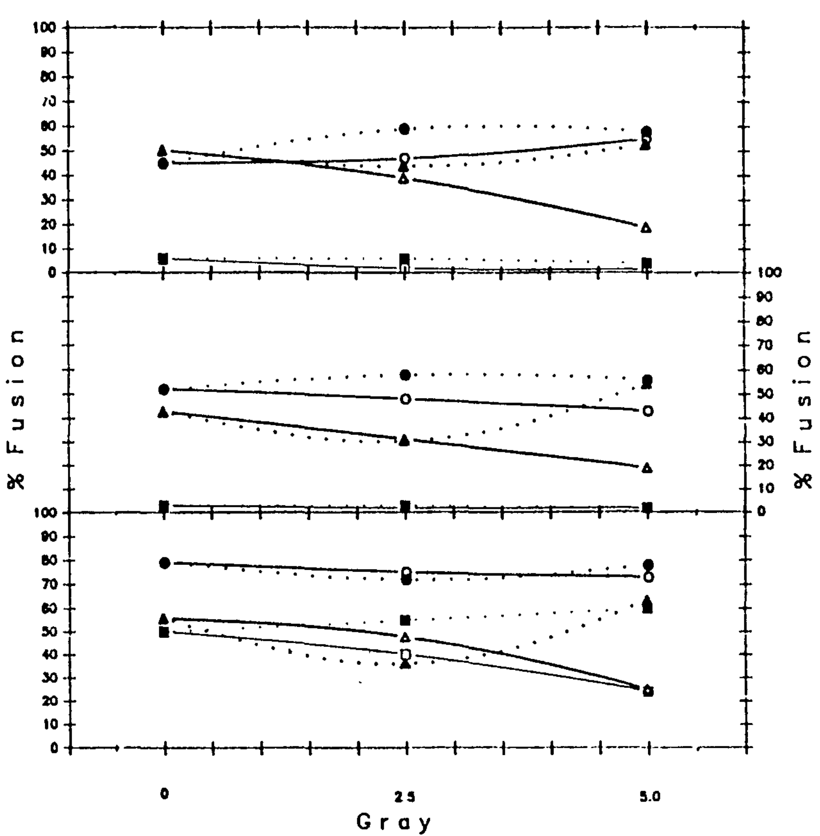
2. Results and Discussion

The extent of cell fusion occurring in cultures of the M9 myoblast clone upon transfer from the "growing medium" to the so-called "fusion medium" with only 1% FCS, was found to be more markedly inhibited by eposure to X-rays if irradiation had been performed at day 1 rather than at day 4 after transfer (Fig. 1, upper panel, open vs. full triangles). The same difference was found also when, in cultures of the same M9 clone, the extent of cell fusion was modified by addition of DZA+HCY (Fig. 1, lowest panel, same symbols). An analogous pattern was also found (Fig. 1, lowest panel, open vs. full squares) in cultures of clone M12 in the presence of DZA+HCY (this clone undergoing practically no fusion in the absence of these drugs). Cultures of clone M6, which fused to a high extent either spontaneously or upon addition of the drugs, were instead almost unaffected, as far as fusion was concerned, by X-irradiation. These findings are consistent with the overall hypothesis that cell commitment to differentiation is inversely related to the susceptibility of the cells to agents interfering with differentiation itself. Cells which had completed commitment, even if they were not yet fused (this event becoming evident only at day 6), were practically insensitive to irradiation, up to 5 Grays.

Figure 1

Effects of X-irradiation on cell fusion in differentiating myoblasts.

Growing myoblasts were transferred to the 'fusion medium' (F14 medium + 1% FCS) additioned with 3 /uM DZA and 50 /uM HCY (lowest panel), with only 50 /uM HCY (middle panel) or without any addition (upper panel), and irradiated either at day 1 (solid line) or at day 4 (dotted line) after transfer. The extent of fusion was always recorded at day 6 after transfer. The results obtained with clones M6, M9 and M12 are indicated respectively by circles, triangles and squares.



The same system of differentiating myoblasts was also investigated by the DNA alkaline elution technique, both to monitor radiation-induced single-strand breaks of DNA and to detect more complex structural differences in chromatin between undifferentiated and differentiated cells. Operationally, cell cultures were pre-labeled for 48 hours with ^{14}C-thymidine, then transferred to the appropriate medium for the desired time and, if required, subjected to X-irradiation, after which they were immediately poured on Nuclepore filters (2 μ porosity). The cells retained on the filters were then lysed with a detergent- and proteinase-containing buffer at pH 9.6, to which a more alkaline (pH 12.2) buffer was progressively added, thus allowing a quantitation of the extent to which a given radiation dose had brought about DNA strand breaks which could be revealed upon alkaline denaturation. The rates of elution of ^{14}C-labeled DNA fragments from the various filters could be converted into $^{14}C/^{3}H$ ratios, thus normalizing the results of different experiments and increasing their overall reproducibility (Sapora et al., in "Radiation carcinogenesis and DNA alterations", F.J.Burns et al. eds., Plenum Press, New York, 1986), by adding to each experimental sample, after irradiation and before the transfer to the corresponding Nuclepore filter, a comparable amount of ^{3}H-labeled undifferentiated cells which had been irradiated at 4.5 Gy (and which served as an internal control). In these experiments, the dose-response curves of the interpolating lines can be assumed to reflect the susceptibility of chromatin DNA to radiation damage, while the Y-intercept indicates to which extent, even without irradiation, chromatin DNA is, in that particular cell system, prone to alkaline denaturation.

In fig. 2 are compared the results obtained with undifferentiated myoblasts -- collected from a "growing" culture -- with those obtained with cells at the 4th or 7th day after transfer to the "fusion medium". In cells collected at day 4 after transfer, the value of the dose-response slope was practically the same as for undifferentiated cells -- their sensitivity to radiation-induced DNA breaks being therefore the same -- but the interpolating line had undergone a significant upward shift, consistent with some "loosening up" of the chromatin architecture. At a later stage of differentiation (day 7 after transfer to the "fusion medium"), the cell DNA was instead more resistant to radiation damage, as indicated by a lower value of the slope of the dose-response curve (fig.2, lowest line), while the Y-intercept had returned to its original value.

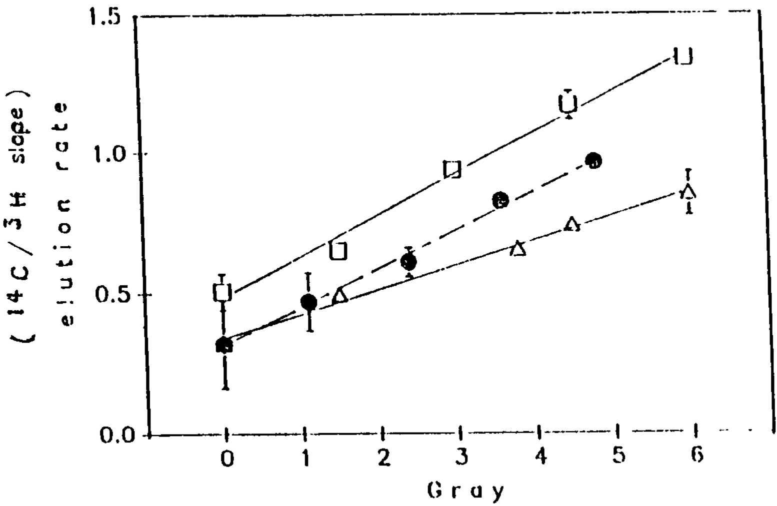
The occurrence of intrinsic (radiation-independent) chromatin rearrangements occurring in the course of the differentiation process can also be evaluated -- apart from the above-mentioned variations of the Y-intercept of fig. 2 -- also by measuring the radioactivity emerging in the unretained fraction which, in these experiments, precedes the actual alkaline elution. The amount of this "unretained" labeled material was negligible in undifferentiated cells, but added up to almost 30% of the whole genomic DNA in the case of differentiating cells

(both at day 4 and at day 7 after transfer), where it consists of acid-precipitable DNA fragments with an average molecular mass of 10^7 Da. Since DNA labeling had been performed before the induction of the differentiation process, it seems reasonable to ascribe the persence of these fragments to the reported occurrence, in rather similar differentiating systems, of enzymatically-induced single-strand breaks correlated to the onset of differentiation (McMahon *et al.*, Proc. Nati . Acad. Sci. USA 81, 7461, 1984).

Figure 2

X-ray dependence of the DNA elution rate induced by alkaline denaturation.

Growing myoblasts, pre-labeled for 48 hours with ^{14}C-thymidine, were subjected to different doses of X-rays either before (closed circles) or after transfer (4 days: open squares; 7 days: open triangles) to the "fusion medium" (F14 medium + 1% FCS). Comparable amounts of cells were then immediately poured on Nuclepore filters, lysed and subjected to progressive alkaline denaturation of their DNA. The rate at which labeled DNA fragments were collected in the eluate is plotted -- after normalization to an internal standard of ^3H-thymidine-labeled cells irradiated with 4.5 Gy -- *vs* the X-ray dose.



The possible effects of variations of DNA methylation on the probability of damage-induced rearrangements in chromatin DNA was also investigated in CHO cells, by studying how the increased frequency of sister chromatid exchange, caused by the two hypomethylating drugs L-ethionine and 5-azacytidine, was actually correlated, through subsequent cell cycles, to the 5-methylcytosine content of nuclear DNA. It was found that, while there was an excellent correlation in the first cycles after addition and removal of the drug, overall DNA methylation levels returned to "normal" values between the 10th and the 16th cycle after removal of the hypomethylating drug, despite the persistence of a high frequency of sister chromatid exchange.

Other experiments were performed with the aim of identifying, in the myoblast system, the existence of an actual correlation between cellular differentiation and variations in site-specific or genome-wide DNA methylation. Two members of this group (D. Carotti and S. Scarpa) have collaborated in 1986 to experiments indicating that, in Friend erythroleukemia cells, a transient hypomethylation (probably due to active removal of methyl groups), followed by remethylation, takes place in the very early stages of differentiation (preceding the first DNA replication) . In order to investigate if active demethylation can indeed be considered as a general phenomenon linked to cell differentiation, and to verify whether it modified the sensitivity to ionizing radiations of the differentiating cells, the levels of DNA methylation were measured throughout the differentiative process of cultured myoblasts. To this purpose, L5 myoblasts in the replicative status (i.e. with 10% foetal calf serum in the culture medium) were labeled for 48h with ^{14}C-uridine. At various times after induction of differentiation by cell transfer to the "fusion medium", chromatin DNA was isolated and hydrolyzed to bases, and the extent of cytosine methylation was measured by HPLC. No significant variation of the methylcytosine-to-cytosine ratio could however be evidenced under these experimental conditions. Even addition of 3-deazaadenosine, known to perturbate S-adenosylmethionine metabolism, was unable to produce appreciable changes in the pre-existing overall DNA methylation levels. A 10% decrease of methyl groups incorporation occurred instead when DNA was labelled after shifting the culture to a "fusion medium" in which 3-deazaadenosine was also present -- this slight hypomethylation remaining constant throughout the entire differentiative process. This moderate inhibitory effect on the methylation of newly-synthesized DNA was however strictly dependent on the presence of the drug, and was apparently unrelated to the differentiation process itself: it did not occur when differentiation was induced by the simple shift to the "fusion medium" in the absence of 3-deazadenosine, nor was it related to the different ability of the drug to accelerate and facilitate the differentiation process in the M6 and M12 myoblast clones. DZA had no effect on the extent of DNA methylation in actively growing cells.

Parallel investigations were performed with the aim of clarifying how, in vitro and in vivo, the DNA methylation process, which is achieved trough the action of a DNA methyltransferase which catalyzes the transfer of the methyl group from S-adenosyl-L-methionine to the 5 position of cytosine moieties present in the DNA, is regulated in eukaryotes. Post-replicative methylation concerns in fact only 3-6% of the cytosines, all of which are reportedly included in the dinucleotide CpG.

A methodological aspect of these investigations concerned the development of a new assay for DNA methylase, based on the release of ^{3}H from the 5 position of selectively labeled cytosine moieties in the DNA -- as an alternative to the standard assay procedure based on the measurement of labeled methyl groups from S-adenosylmethionine to acid-precipitable DNA. In order to validate this new method, it was verified whether DNA-methyltransferase-catalysed transfer of methyl groups from S-adenosylmethionine to the 5 position of cytosine moieties of DNA was in exact stoichiometry with the liberation of the hydrogen atom from this same position of the cytosine moieties. Since in early experiments tritium release was always found to be greater than apparent methyl transfer, the interesting possibility arose, that the methylase was catalysing an exchange reaction at the 5-H position of DNA cytosine in addition to the methylation reaction itself. A source of experimental error found in the standard (traditional) assay was the impurity of commercially available S-adenosylmethionine, as well as, occasionally (when Micrococcus luteus DNA was tested for de novo methylation), inefficient trichloroacetic acid precipitation of the DNA. After correction for these errors, good agreement was found between the two methods, using either ^{3}H-dCTP nick-translated mammalian DNA, as a hemimethylated substrate, or Micrococcus luteus DNA for de novo methylation. Various types of DNA were also compared as substrates. The best for efficiency and for other practical reasons was nick-translated (and therefore partially hemi-methylated) mammalian DNA. The amount of ^{3}H which was released (measured on the basis of original label present in the DNA) was found to be independent of the extent of nick translation both in hemimethylation and de novo methylation. By adding, before or at various times during the reaction, unlabeled DNA which was otherwise chemically identical with the labeled substrate, it was also possible to investigate the processivity of the reaction. The time of addition of the non-radioactive substrate was found to make no difference as far as the rate of ^{3}H release was concerned, this result being evidence against a processive mechanism.

As a further approach to the problems of the regulation of DNA methylation, polydeoxyribonucleotides with known base composition were synthesized, in order to investigate the enzyme requirements in terms of bases adjacent to the CpG target. As enzyme source human placenta was used. The presence of guanine nucleotides, and in particular of the dinucleotide CpG, seemed the only necessary

characteristics required by these synthetic polymers in order to be substrates in the methylation reaction. The presence of a methylcytosine on the opposite strand greatly favoured the reaction.

In the course of the reported investigations an effect of DNA concentrations on methylase activity was observed. High concentrations (above 200 μg/ml) of double-stranded Micrococcus luteus DNA were found to induce a time-dependent inhibition of the 'de novo' methylase activity, while single-stranded DNA did not show such an effect. The enzyme was apparently irreversibly inhibited because neither high salt dissociation of the DNA-enzyme complex nor extensive digestion of the inhibitory substrate could restore the initial rate of methylation. The inhibitory effect seemed to be related to the base composition of the substrate, because it was not found with the synthetic polymer poly(dI-dC).poly(dI-dC), while it was present using either poly(dC-cG).poly(dC-dG) or poly(dA-dT).poly(dA-dT).

As a natural prosecution of these experiments we have also studied the influence of the average distance between CpG's on the efficiency of catalysis. This choice was essentially motivated by a peculiar characteristics of vertebrate DNA: although the entire genome is generally CpG-depleted, peculiar areas exist, in which the CpG dinucleotides are present at a higher frequency (similar to that of the GpC dinucleotide), but in an unmethylated status. These so-called CpG-rich islands are always found at the 5' end of housekeeping genes and seem to have a role in controlling the expression of the associated genes. How these regions, which should theoretically behave as excellent substrates for the enzyme, can escape methylation in vivo is still an open question. We have analyzed the in vitro methylation pattern of several plasmids containing cloned genomic DNA sequences of widely different CpG frequencies, ranging from an average of 1 CpG every 8 nucleotides, as found in CpG-rich islands, to 1 CpG every 200 nucleotides, typical frequence in a random genomic DNA. Human placenta enzyme was used with cloned human and murine CpG-rich islands and with the human beta-globin gene.

The extent of methylation for a single sequence was measured after restriction digestion of an in vitro methylated plasmid and subsequent isolation of the fragments by means of agarose gel electrophoresis. The amount of radioactivity incorporation was correlated to the number of CpG's present in each fragment. The correlation was very good for all fragments except for those containing an average CpG spacing lower than 11 nucleotides. In this case the methylation efficiency was reduced of about 40%. In our analysis isolated CpG's, generally considered as less favourable substrates, become methylated to the expected level. The system seems to reflect the physiological situation where isolated CpG's are almost completely methylated and clustered ones are not. Eukaryotic DNA methyltransferase shows, therefore, a peculiar sensitivity to CpG

frequency, and this characteristics can be expiected to play a regulatory role in maintaining CpG-rich islands in a hypomethylated condition.

These results do not however totally account for the "physiological" in vivo situation, where practically no methyl groups are usually found in clustered CpG's. This discrepancy between the in vivo situation and the in vitro results suggests that, in vivo, interaction with other chromatin component(s) can also play a role in preventing methylation of clustered CpG's -- but no direct experimental evidence supporting this hypothesis has sofar been obtained. Experimental data concerning the influence of chromatin structure (and of its different components) on the activity of eukaryotic DNA-methyltransferase have instead shown that, at least in human placenta chromatin, there are some tightly-bound components which strongly stimulate, in vitro, the enzyme-catalysed transfer of methyl groups to DNA cytosine moieties. It was also found, as an in vivo counterpart of this phenomenon, that, in chromatin loops, there are some DNA segments characterized by a high methylation level and by a strong association with some "tightly-bound proteins".

Within the general framework of collaborations with other research groups, new kinetic methodology was developed, through which novel results were obtained about interactions between allosteric effectors in deoxycytidylate aminohydrolase, including demonstration that occupation of activator sites by substrate gives rise to extremely high cooperativities. Stoichiometry of substrate and modifier sites of dCMP aminohydrolasre was established, and its consequences for allosteric mechanisms were analysed. Half-site reactivity in the enzyme glutathione-S-transferase was also studied.

IV. Other research group(s) collaborating actively on this project - name(s) and address(es):

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Dr. D. Toniolo; Istituto Internazionale di Genetica e di Biofisica del C.N.R., Napoli (Italy).

Dr. H.-P. Saluz; Friedrich-Miescher Institut, Basel (Switzerland).

V. PUBLICATIONS:

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: B16-D-177-I

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Head(s) of research team(s) [name(s) and address(es)]:

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Title of the research contract:

Radiation carcinogenesis in vitro.

List of projects:

1. Radiation carcinogenesis in vitro.

Title of the project no. 1: **Radiation Carcinogenesis in vitro**

Head of Project and Scientific Staff: L. Tallone Lombardi, D. Bettega, P. Calzolari, A. Ghidoni, A. Ottolenghi.

The general aims of this project were to obtain experimental data on the biological effectiveness and dose-responses of various types of radiation, and to use these quantitative data to test models of radiation action and seek biologically relevant parameters of radiation quality.

The experimental work was mainly centred on determining the dose-effect relationship for induction of oncogenic transformation in cultured mammalian cells exposed to low doses of high LET ionizing radiations such as alpha particles from radioactive decay. In addition cell survival, growth kinetics and cell density effects after exposure to various type of radiations, were also determined.

The system used was the C3H10T1/2 mouse embryo fibroblast cell line. The cells were cultured following standard protocols and transformation frequency was determined by the number of type II and III foci following the criteria already established in the literature. Radiations used were low LET protons from a Cyclotron, 220 KVp X-rays and α-particles from a ^{244}Cm source.

Oncogenic transformation

Our main activities centred on studying dose-effect relationship in transformation induced by 4.3 MeV α-particles,with an LET of 101 $KeV/\mu m$. For this purpose, cells were cultured on a 3 cm diameter, 1.5 μ thick mylar foil sealed with araldite to a pyrex ring. Using this support no difference was found in growth rate, plating efficiency or spontaneous transformation frequency as compared with cells cultured on conventional Petri-dishes. The samples were irradiated in a purpose-built irradiation chamber. Survival and transformation were determined in parallel and were compared with analogous results obtained with low LET protons. The survival curve with alpha was an exponential function of the dose with a mean lethal dose of 0.61 ± 0.02 Gy. The corresponding RBE was a decreasing function of the dose varying from 15 to 4 when the alpha particle doses ranged from 0.1 to 2 Gy. The transformation curves showed complex shapes with an apparently constant region at low doses followed by a linear increase with dose. RBE values decrease from 20 to 4 in the dose region between 0.1 and 2 Gy. There was a significant transformation incidence at doses that have very little effect on cell survival. No effect of the dose rate was found at total doses of 0.05 or 0.1 Gy with dose rates of 0.11 and 0.005 Gy/min.

The effect of the temporal distribution of the dose was also studied with low LET protons (1.82 ± 0.03 $KeV/\mu m$). Total doses of 0.5,1 and 7 Gy were delivered as a single fraction and two equal fractions with various time intervals up to 10 h between doses. The frequency of transformation per surviving cell is lower with split doses than with single doses by factors of 3.1 ± 0.5, 3.1 ± 0.6 and 1.5 ± 0.3 at doses of 7, 1 and 0.5 Gy respectively.

Cell-density effect

Cell density dependence of transformation frequency was studied in C3H10T1/2 cells exposed to 0.5 and 7 Gy of 200 KVp X-rays in the cell density range between 50 and 2500 cells/10 cm diameter Petri-dish. It was found that the initial cell density strongly influences transformation frequency with decreasing factors of about 4 and about 10 at 7 and 0.5 Gy respectively throughout the density interval studied. The data can be well fitted with two equations: an allometric function represented on a log-log scale by a straight line and a sigmoidal function with two plateaux between 50 and 250 cells/dish and above 600 . The region between 50 and 250 cells/dish turned out to be a region of low variation in transformation frequency values. Similar conclusions have been reached in three out of four cases from an analysis of data reported in the literature.

Cell growth kinetics

Growth curves and colony sizes were determined in C3H10T1/2 cells exposed to 0, 1, 3, 5 and 7 Gy of low LET protons. The data were analyzed to determine the temporal dependence of the growth properties of survivors and to investigate whether the surviving percentages are well represented by the conventionally calculated surviving fractions.

The data showed that irradiated populations could be represented by a moiety of surviving cells which have the same growth rate as the unirradiated cells and a moiety of non-surviving cells which begin to divide at nearly the same growth rate but gradually lose their ability to divide. Growth curves were studied with a model represented by an exponential plus a Gompertz function which takes into account the behaviour of the surviving and non surviving subpopulations. It was found that the surviving cells grow at a constant rate $a = 0.029 \pm 0.002\, h^{-1}$; non-surviving cells have a growth rate equal to a, but lose their ability to divide at a rate per unit of dose $b = 0.0041 \pm 0.0009\ h^{-1}\, Gy^{-1}$; division delay was short,being equal to $1 \pm 0.8\ h\, Gy^{-1}$. Growth curves of the entire population are influenced by non-survivor progeny up to 150, 200 and 250 h from irradiation at 3,5 and 7 Gy respectively; at longer times the population can be considered to consist of survivor progeny.

The percentage of cells having the same growth rate as the control population is less by a factor of about 2 than that of survivors calculated from macrocolony counting at doses as high as 7 Gy.

Survival models

Survival curves of C3H10T1/2 cells reported in the literature were analysed by using multitarget, multihit and linear quadratic models. The relative parameters were analyzed and the variation between the results obtained by different laboratories discussed. The inactivation probability density function for each curve was studied and the mean lethal dose, variance and mode of distribution calculated. A set of equations was also proposed , which greatly simplify the calculation of these quantities.

The main conclusions of this study were :

a) The best parameters to represent cell survival are the mean $\overline{D}$ and the standard deviation σ of the inactivation probability density function. The values of parameters, α, β, D_0, n, k, and λ are more variable from experiment to experiment and more dependent on the range of available data used for estimation.

b) The quasi threshold dose Dq is the mode of inactivation probability density. It can be also interpreted as the dose around which the cell response to a dose increment is at its maximum.

Publications

1. D. Bettega, P. Calzolari and L. Tallone Lombardi Split-dose effects in C3H10T1/2 exposed to low LET radiations. 20th annual meeting of the European Society for Radiation Biology Pisa Sept. 15-19 1986, in Int. J. Radiat. Biol. 51, 933 (1987).
2. D. Bettega, P. Calzolari and L. Tallone Lombardi Radiocarcinogenesis: results from in vitro experiments. Il Nuovo Cimento, 9D, 1205-1217 (1987).
3. D. Bettega, P. Calzolari and L. Tallone Lombardi Cell Density Effect on Transformation. (8th International Congress of Radiation Research. Edimburgh 19-24 July (1987).
4. D. Bettega, P. Calzolari and L. Tallone Lombardi Effects of split-dose irradiation on survival and oncogenic transformation induced by 31 MeV protons in C3H10T1/2 cells. Int. J. Radiat. Biol. 52, 761-765 (1987).
5. D. Bettega, P. Calzolari, A. Ottolenghi and L. Tallone Lombardi Growth Kinetics of C3H10T1/2 exposed to low LET radiations. Int. J. Radiat. Biol. 55, 641-651 (1989).
6. D. Bettega, P. Calzolari, A. Ottolenghi and L. Tallone Lombardi Transformation of C3H10T1/2 cells with 244Cm alpha particles at low and high dose rates.in Cell Transformation and Radiation- induced Cancer (K.H Chadwick, C Seymour and B.Barnhart eds.) pp.333-340,Adam Hilger, Bristol and New York, (1989).
7. D. Bettega, P. Calzolari, A. Ottolenghi and L. Tallone Lombardi Oncogenic transformation induced in vitro by radiation of varying LET. Radiation Protection dosimetry. Accepted for publication (1989).
8. D. Bettega, P. Calzolari, A. Ottolenghi, E. Rimoldi and L. Tallone Lombardi. Cell density effect of transformation frequencies in C3H10T1/2 exposed to X-rays. Int. J. Radiat. Biol., 56, 989-998 (1989).
9. D. Bettega, P. Calzolari, A. Ottolenghi, and L. Tallone Lombardi. Modelli di interazione della radiazione ionizzante con sistemi cellulari. Invited paper. V Congresso S.I.R.R. Roma 12-14 ottobre (1989). In press.
10. D. Bettega, P. Calzolari, A. Ottolenghi, and L. Tallone Lombardi. Carcinogenesi da Radiazione. Invited paper. XVIII Congresso A.I.R.B. Napoli 21-23 Settembre (1989). In press.
11. D. Bettega, P. Calzolari, A. Ottolenghi, and L. Tallone Lombardi. Criteria and techniques for analysing cell survival data. Submitted for publication (December 1989).

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-091-D

Kernforschungszentrum
Karlsruhe GmbH
Postfach 3640
D-7500 Karlsruhe 1

Head(s) of research team(s) [name(s) and address(es)]:

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Title of the research contract:

The fractionation and speciation of plutonium and other actinide elements in vivo.

List of projects:

1. The fractionation and speciation of plutonium and other actinide elements in vivo.

Title of the project no.: B16-D-091-D

The fractionation and speciation of plutonium and other actinide elements in vivo.

Head(s) of project: Prof. Dr. D. M. Taylor

Scientific staff:

Prof.Dr. V.Volf	Prof.Dr. A.Seidel	Dr. F.Planas-Bohne
Dr. W.Rau	Dr. J.R.Duffield	Dr. W.-G.Thies
Dr. M.Neu-Müller	Dr. R.Wirth	S.A.Proctor, BSc
L.Yule, BSc	L.C.Farrow, BSc	Dipl.Chem. B.Köhler
Dipl.Biol.U.Schuppler	E.A. Davies	P.Unalkat

I. Objectives of the project:

1. To study the chemical forms in which plutonium and other actinides are present in blood plasma, in intracellular structures, such as lysosomes, and in the various compartments of the gastrointestinal tract.

2. To elucidate the mechanisms of transfer across plasma and mucosal membranes and to identify factors which may influence the transfer.

II. Objectives for the reporting period:

a. To study the role of transferrin as the principal transport protein for actinide and lanthanide elements in blood plasma.

b. To examine the cellular uptake of metal-transferrin complexes in relation to the transferrin receptor mechanism.

c. To investigate the binding of actinide metals to intracellular ligands.

d. To develop an experimental model of the human gastrointestinal tract for the purposes of examining the speciation of actinides and identifying factors which may influence their absorption into the blood stream following ingestion.

III. Progress achieved:

a. Binding of actinides to transferrin.

Transferrin is the principal iron transport protein in mammalian blood plasma, but previous studies from this and other laboratories have shown that plutonium, thorium, americium and curium are also transported in the blood as transferrin complexes. During the course of this project the binding of the actinides neptunium-239, protactinium-233, and the lanthanides ytterbium-169 and europium-154 to serum proteins has been investigated in vivo, following intravenous injection of the metal citrates into rats, and in vitro in rat, horse or human serum.

The binding of the radionuclides was examined by sequential gel permeation chromatography on Sephacryl S-300 followed by re-chromatography of the co-eluted albumin-transferrin fractions on DEAE-Sepharose, or DEAE Cellulose, eluting with a linear gradient of 0 to 0.5 M NaCl. The results from the studies with neptunium, protactinium, ytterbium and europium showed that transferrin was the principal binding protein in blood serum after both in vivo and in vitro labelling. The association of the actinide or lanthanide with transferrin was further verified by means of polyacrylamide gel electrophoresis and by immunodiffusion methods. Saturation of the serum with iron led to the displacement of plutonium, neptunium and ytterbium from the transferrin complex, providing strong evidence that the metals bind to the two iron-binding sites in the transferrin molecule. Protactinium was not displaced by iron saturation of the serum suggesting that either the protactinium-transferrin complex has a greater stability than the iron-transferrin complex, or that this metal does not bind to the iron-binding sites. Ultra-violet difference spectroscopy of the plutonium- and ytterbium-transferrin complexes, formed from purified human apo-transferrin, indicated that the metal binding capacity of human transferrin was two metal atoms per protein molecule, the same as for iron. Attempts to demonstrate the formation of the Pu-Tf and Pu_2-Tf complexes using iso-electric focussing electrophoresis were unsuccessful due to the dissociation of the metal protein complex during electrophoresis; this technique clearly demonstrates the Fe-Tf and Fe_2-Tf complexes, thus it seems likely that the plutonium-transferrin complex is less stable than that with iron. Ytterbium may be regarded as an analogue for a trivalent actinide, and americium and curium have been shown previously to form relatively weak complexes with transferrin. In collaboration with the School of Chemistry and Applied Chemistry of the University of Wales College of Cardiff, computer simulation methods for the calcula-

tion of metal speciation in biological systems such as blood plasma, the gastrointestinal tract and in actinide-contaminated wounds have been introduced into this Institute and applied to various problems. One example was the use of computer simulation methods to calculate a minimum value for the formation constant, log ß, of the ytterbium-transferrin complex; this calculation yielded a value of about 12, some 10 to 15 orders of magnitude less than that of the iron-transferrin or plutonium-transferrin complexes. In summary this phase of the work confirmed the importance of transferrin as the principal transport protein for actinides, and for at least two lanthanides, in mammalian blood plasma in vivo. The studies further suggest that the two iron-binding sites on the transferrin molecule are important for the binding of the actinide and lanthanide metals. This was confirmed in other studies which have demonstrated that, as for iron, bicarbonate is required as a synergistic ion for metal binding to take place. Studies still in progress are concerned with the estimation of the thermodynamic stability constants for the formation of the plutonium-transferrin complex.

In collaboration with the Institute of Experimental Nuclear Physics of the University of Karlsruhe, attempts are being made to elucidate the mechanisms of binding of foreign metals to transferrin using hafnium as a plutonium analogue and europium as an americium analogue and various spectroscopic methods including UV-spectroscopy and time-dependent perturbed angular correlation of gamma rays. These investigations confirm the importance of the iron-binding sites and have indicated that phosphate ions, as well as bicarbonate ions, may be involved in the metal binding. In further collaboration with the Daresbury Laboratory in the UK, attempts are being made to apply x-ray small angle scattering to the analysis of complexes in solution, with the aim of obtaining detailed information of the binding of the metals to the N- and C- terminal binding sites in the transferrin molecule.

b. The cellular uptake of the iron- and plutonium-transferrin complexes.
There is considerable experimental evidence to indicate that iron is taken up into at least some mammalian cell types via a receptor mediated mechanism involving a transferrin-specific receptor. In view of the observations discussed above which show that actinide and lanthanide metals bind to transferrin after entry into the systemic circulation of animals, it was of interest to investigate whether plutonium was taken up into cells via this

transferrin-receptor mechanism. In a large series of experiments using a range of human or animal cells in culture the uptake of iron-59 or plutonium-239 from either the citrate or transferrin complexes was compared following incubation of the cells with the metal complexes in vitro.
Studies of the total uptake of iron-59 by four cell lines, primary cultures of rat hepatocytes, and the human cell lines Hep G-2 Hepatoma, HeLa, and WI-L2 Lymphoblasts, showed that the uptake of iron from the citrate complex was greater by factors ranging, according to cell type, from 8 to 100 than that from the transferrin complex. Essentially similar results were obtained in the studies with plutonium-239, except that the differential between the uptake from citrate and from transferrin was smaller, ranging from about 1 with Hep G-2 cells to about 10 in primary rat hepatocytes. More detailed studies were made to distinguish between membrane-bound and truly intracellular metal, these showed that both iron-59 and plutonium-239 were bound to the cell membrane in a form which could be released by a brief treatment with trypsin. This suggested the involvement of a metal binding protein or receptor in the cell membrane. The amount of the iron-59 bound to the cell membrane was always smaller than the amount transferred to the interior of the cell, however, for plutonium membrane binding exceeded cellular uptake by factors ranging from about 3 for the citrate to about 40 for the transferrin complex. Studies in rat hepatocytes using a number of different types of metabolic inhibitor suggested that neither the intracellular accumulation nor the membrane binding of either iron or plutonium were influenced by clustering of membrane proteins, intactness of microtubuli or of microfilament assembly; further endocytosis did not appear to play an important role in the uptake of these metals by hepatocytes.
As part of a search for in vitro models for human or animal organs for studies of the mechanisms of metal uptake and loss liver spheroids, clusters of 200 to 300 cells, were prepared from pure, freshly isolated, rat hepatocytes and from mixtures of hepatocytes, endothelial cells and Kupffer cells and compared in relation to the uptake of plutonium and iron and to the retention of biochemical parameters, such as the enzymes GOT, GPT and Cytochrome P450 and protein synthesis, with monolayer or single cell suspensions of liver cells. The results suggested that while the liver cell spheroid is an excellent system for the study of the metabolism of organic substances its applicability to inorganic biochemical studies, especially to metal speciation analysis, is limited.
These comparative investigations of the role of the metal-transferrin

complex-transferrin-receptor system in the uptake of iron and plutonium suggest that for primary rat hepatocytes, rat liver cell spheroids and for other cell lines such as HeLa, iron does appear to be incorporated into the cell via this mechanism, although it may not be the major uptake mechanism in all circumstances. With plutonium although large amounts are bound to the cell membrane transfer to the cell interior is very small when transferrin is present in the medium, thus the transferrin-receptor mechanism does not appear to be important in facilitating the transfer of plutonium into the cell, indeed, the presence of transferrin may be a form of detoxication mechanism hindering the uptake of this foreign metal into the cell. It has not yet been established unequivocally whether the plutonium-transferrin complex binds irreversibly to the transferrin receptor on the cell surface, or whether the membrane binding of plutonium involves a site other than the transferrin receptor. If irreversible binding of the plutonium-transferrin complex to the transferrin receptor does occur this would indicate strongly that the binding of plutonium to transferrin results in some conformational change which inhibits dissociation of the metal-transferrin-receptor complex. The aim of the studies discussed under (a) above is to attempt to establish if such conformational changes do occur, not only with plutonium but also with other "foreign" metals which form stable complexes with transferrin in vivo.

c. The subcellular distribution of actinides in liver.

Previous studies have shown that following entry into the systemic circulation plutonium, americium, curium and actinium deposit predominantly in lysosomal structures in liver and that the iron-storage protein ferritin appears to be a final storage form. The subcellular distribution studies carried out during this contract were concerned with three topics: the uptake of plutonium into liver cell nuclei; the subcellular distribution of neptunium and protactinium; and the mechanism of transfer of plutonium and protactinium from the liver cell membrane to the lysosomes. Autoradiographic studies in liver specimens obtained from rats and hamsters following intravenous injection of plutonium-239 indicated the deposition of a small but significant fraction of the total cellular plutonium in the cell nucleus. Since such nuclear deposition could be important in relation to the induction of radiotoxicity a comparative investigation of the cellular uptake and distribution of plutonium-238 and plutonium-239 in 2-day old cultures prepared from freshly isolated rat hepatocytes was carried out. The cells were

incubated for periods of up to 5 hours in serum-free Waymouth's MB752/1 medium containing plutonium citrate, 670 nM Pu-239 or 2.5 nM Pu-238; after incubation the cells were washed thoroughly in Hank's balanced salt solution suspended in 0.25 M sucrose-5mM Tris.HCl pH 7.4 and homogenised by passage through a 10 micrometre pore-size polycarbonate membrane filter. Standard centrifugal methods were then used to obtain the lysosomes, other subcellular organelles and purified nuclei. The results showed that more than 70% of the cellular plutonium-238 and 55% of the plutonium-239 was deposited in the lysosomes. Nuclear deposition of plutonium-239 ranged from 10% after 1 hour to 30% after 5 hours of incubation, in contrast plutonium-238, which has a 270-fold greater specific activity showed no significant association with the cell nuclei. These results suggest that nuclear uptake of plutonium is mass dependent; it appears unlikely to be of real significance for the radiological protection of workers in the nuclear industry, except in the case of a very severe internal contamination incident.

Since data on the subcellular distribution of neptunium and protactinium were not available in the literature, limited studies of the subcellular distribution of these elements in rat liver following intravenous injection of either neptunium-237, neptunium-239 or protactinium-239 as their citrates were made. The results obtained using standard differential centrifugation methods or free-flow electrophoresis for subcellular fractionation showed that, like the other actinides studied, both elements were deposited predominantly in lysosomes.

In order to try to learn something about the mechanisms of transfer of plutonium and protactinium from the cell membrane to the lysosomal structures in liver cells the time dependent distribution of the actinide-binding components was studied in liver cytosol, prepared from either in vivo or in vitro labelled cells, using gel chromatography on Sephadex and Sephacryl gels. The results for both plutonium and protactinium indicate that very rapidly after entry through the cell membrane the metals become bound to proteins and to low molecular weight ligands in the cytosol, one protein of molecular weight about 200 kD appears to be specially important in that over the course of about 12 hours protactinium and plutonium appear to be transferred almost quantitatively from this protein to ferritin, the predominant storage protein for actinides in the cell. This 200 kD protein appears to play no role in the transfer of iron from cell membrane to ferritin, the protein itself, which appears to be present in only very small amounts, has not yet been characterised. Some studies have been carried out to at-

tempt to elucidate the way in which plutonium becomes incorporated into ferritin, the preliminary results have led to the proposal of the hypothesis that the plutonium atoms become lodged in the channels in the apoferritin core, considerable further work will be required to substantiate this hypothesis.

d. The speciation of actinides in the gastrointestinal tract.
Studies in experimental animals, plus very limited studies in man, suggest that the absorption of plutonium and other actinide elements from the human gastrointestinal tract is very small, probably much less than 0.1% of the ingested amount. However, the animal studies indicate clearly that absorption can be influenced by physiological factors such as fasting as well as by dietary components and by some drugs. The study of absorption at these very low levels in experimental animals is difficult, requires many animals and often yields results with large experimental errors, further a very large and expensive experimental programme would be required to investigate all the physiological, pathological and food factors which could lead to enhanced absorption of actinides in man. In order to attempt to elucidate the chemical speciation of actinide elements in the various compartments of the human gastrointestinal tract a simple in vitro model has been developed and used to study the fractionation of plutonium, neptunium and protactinium and the ways in which the fractionation is influenced by various types of foodstuff.
The model consists of a "mouth" in which reactions with human saliva may be studied, after the required reaction time a sample is removed for analysis and to the remainder a simulated gastric juice, with a similar enzyme and salt composition to human gastric juice, is added and allowed to react, after sample removal simulated duodenal juice is added to the reminder of the gastric system and after an appropriate reaction time a further sample is removed. In the initial studies the samples were analysed by centrifugation to separate insoluble and soluble material, the soluble fraction was then analysed by chromatography on Sephadex G-50 to yield "protein-bound", "low-molecular weight soluble" and "hydrolysed" fractions.
Figure 1 illustrates the results obtained with two important dietary components, tea and orange juice, for plutonium-239, neptunium-239 and protactinium-233; the data show clearly that for all three actinides the proportions of insoluble, hydrolysed, low molecular weight and protein bound nuclide change markedly during the transition from mouth to stomach to d

denum. This suggests that the components of the diet ingested with the actinide are likely to play a greater role in determining its chemical speciation in the gastrointestinal tract than the actual chemical form in which the actinide was ingested. Thus tea results in predominantly insoluble, and presumably non-absorbable, material while the citrate in orange juice leads to a high proportion of soluble low-molecular weight complexes, which may

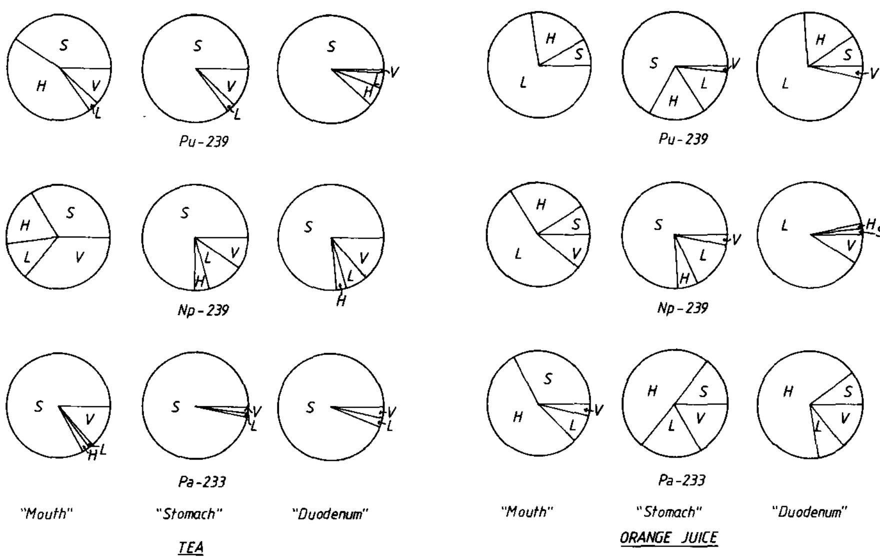

Figure 1. The percentage distribution of the chemical fractions of plutonium, neptunium and protactinium in the simulated human gastrointestinal tract. S - Sedimentable (insoluble) material; V - Protein-bound material; L - Low molecular weight soluble species; H - "Hydrolysed" species.

Following these simple initial studies attention has been concentrated on the "duodenum", the compartment from which absorption through the mucosal wall into the systemic circulation occurs. More refined methods for the analysis of the low molecular weight fraction from the simulated duodenum, using cation and anion exchangers and solvent extraction were developed. These studies suggested that following introduction of plutonium nitrate or plutonium citrate into the mouth in the absence of other dietary factors, the soluble low molecular weight fraction in the duodenum was about 17% and 70-80% respectively of the total nuclide. However, for each compound only

about 0.1% was present in the form of electrically neutral species which might be expected to be adsorbable. Computer speciation analysis of plutonium in a duodenal system containing citrate also suggests that about 0.1% was present as uncharged complexes. It is important to note that the in vitro model and the computer simulation yield values for the potentially absorbable plutonium fraction which lie close to those for the absorption of plutonium from citrate observed in experimental animals.

Most recent studies have involved the addition of a simulated bile to the duodenal system and the systematic examination of the influence of endogenous components of the duodenal fluids on the speciation; of particular interest has been the observation that lecithin may play an important role in controlling plutonium speciation. Lecithin vesicles formed in the duodenum under physiological conditions can trap appreciable amounts of plutonium, or other heavy metal, in a form which appears likely to be non-transportable through the cell membrane. The possibility of developing a lecithin vesicle therapy for the limitation of actinide absorption in the event of an oral contamination incident is being investigated.

e. General Discussion

The four topics discussed above have all been concerned with improving understanding of the chemical mechanisms involved in the transport of actinides in blood, across cell membranes and within the cell itself from the site of entry to the ultimate deposition site. The elucidation of these mechanisms is of fundamental importance in relation to our understanding of actinide and general heavy metal biochemistry and of the reasons why the specific metals assume their particular distribution patterns in the body. Further such knowledge assists in the understanding and prediction of toxic effects in man.

From the point of view of radiation protection, apart from their contribution of our fundamental understanding of the metabolic behaviour of the highly radiotoxic actinide elements, the data discussed above have two practical applications.

The studies of actinide binding to transferrin and the subcellular ligands are important in relation to the development of improved methods of chelation therapy for accelerating the removal of plutonium and other actinides from the human body following an internal contamination incident. These studies show that an essential requirement for any new chelator for the treatment of plutonium contamination in man is that it must mobilise

plutonium from its transferrin complex in the blood, and release the metal from ferritin in the cells. Thus in vitro studies of the ability of any proposed new chelator to mobilise the chosen actinide from its transferrin and ferritin complexes are now an essential pre-requisite for the testing of the substance in animals. During the period under review in vitro studies of the ability to release plutonium from transferrin and ferritin have been an essential part of our screening programme for new chelators and this has led to a considerable reduction in the number of animal experiments carried out.

The use of in vitro models and of computer simulation methods for the study of the chemical speciation of metals in the gastrointestinal tract has already been developed to the point where predictions about factors in the human diet which may either lead to enhanced actinide absorption from the gastrointestinal tract, or which can be recommended for limiting absorption, can be made with reasonable confidence. Further development of these methods, coupled with their validation by a few very carefully designed and executed studies in experimental animals or in human volunteers, can be expected to increase the confidence with which predictions can be made and eliminate the need for a very large programme of studies in experimental animals.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

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Daresbury Laboratory, Warrington, WA4 4AD, U.K.
European Late effects of Radiation Project (EULEP).

V. Publications:

Wirth, R., Taylor, D.M., Duffield, J.R.. Identification of Transferrin as the Principal Neptunium-binding Protein in the Blood Serum of Rats. International Journal of Nuclear Medicine and Biology, 12, 327-330, 1985.

Taylor, D.M., Duffield, J.R., Proctor, S.A.. The Chemical Forms of Plutonium in the Gastrointestinal Tract. In: R.A.Bulman and J.R.Cooper (Eds). Speciation of Fission and Activation Products in the Environment. pp.208-212, 1986. Elsevier Applied Science Publishers, London-New York.

Schuler, F., Taylor, D.M.. The subcellular distribution of Pu-239 in primary cultures of rat hepatocytes. Radiat. Res. 110, 362-371, 1987.

Taylor, D.M., Farrow, L.C.. Identification of Transferrin as the main binding Site for Protactinium in Rat Blood Serum. Nucl. Med. Biol. 14, 27-31, 1987.

Taylor, D.M., Seidel, A., Planas-Bohne, F., Schuppler, U., Neu-Müller, M., Wirth, R.E.. Biochemical Studies of the Interaction of Plutonium, Neptunium and Protactinium with Blood and Liver Cell Proteins. Inorg. Chim. Acta 140, 361-363, 1987.

Duffield, J.R., Taylor, D.M.. A Spectroscopic study on the binding of Plutonium (IV) and its chemical analogues to Transferrin. Inorg. Chim. Acta 140, 365-367, 1987.

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Planas-Bohne, F., Duffield, J.R.. Factors influencing the uptake of iron and plutonium into cells. Int. J. Radiat. Biol., 53, 489-500, 1988.

Schuppler, U., Planas-Bohne, F., Taylor, D.M.. Biochemical binding and distribution of protactinium-233 in the rat. Int. J. Radiat. Biol. 53, 457-466, 1988.

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Taylor, D.M., Thies, W.-G., Appel, H., Neu-Müller, M., Schwab, F. and Weber, H.W.. Spectroscopic studies of Hafnium-Transferrin-Complexes: Differences between the two binding sites. Metal Ion Homeostasis: Molecular Biology and Chemistry, Alan R.Liss, Inc. pp.179-188, 1989.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor:

Trinity College
IRL-Dublin 2

Contract no.: BI6-D-184-IRL

Head(s) of research team(s) [name(s) and address(es)]:

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Telephone number: 01-772941

Title of the research contract:

Interaction between radiation and environmental carcinogens, studies on human cells in vitro.

List of projects:

1. A study of the transformation of primary human cell cultures by radiation and radiation in combination with environmental mutagens.

Title of the project no.: B16-D-184-IRL

Head(s) of project: Dr K F Tipton
Dr C B Seymour
Dr C Mothersill

Scientific staff: Dr K F Tipton
Dr C B Seymour
Dr C Mothersill
Mr J O'Rourke
Ms M McDonnell
Ms A Cusack

I. Objectives of the project:

1. To use primary cultures of human tissues to study the interaction between radiation and known or suspected environmental carcinogens, promotor's, mutagens or toxins.

2. To design model systems where a carcinogen's effect in its _in vivo_ target organ can be studied in relevant systems _in vitro_.

3. To screen treated cells for changes in ultrastructure or iocenzyme profile in addition to conventional transformation endopints which often have to be studied in specialised cell lines or rodent models.

II. Objectives for the reporting period:

1. To quantify increased proliferation of epithelial cells resulting from combined exposure to radiation and carcinogens.

2. To quantify as far as possible the intermediate filament and biochemical changes.

3. To investigate the problem of metabolic activation of carcinogens in culture and to see what role irradiation might have in this.

4. To continue to study the effects of tumour promotors on the system.

5. To continue epidemiological studies to identify risk factor combinations.

III. Progress achieved:

INTRODUCTION

It has become apparent in the last few years that in animals and cell culture systems some environmental carcinogens can have interactive affects with radiation and many of these are supra-additive. Many of the mechanisms involve a carcinogen which is activated by radiation or which yields high numbers of toxic free radicals when irradiated. Other carcinogens are thought to destabilise the DNA making radiation damage more prone to misrepair

The implications of supra-additive effects of environmental carcinogens and radiation for radiation protection are that doses which on their own can be shown to be harmless may have adverse effects in a real situation where low levels of environmental carcinogens may be present.

The contract sought to approach this problem on two levels, first by developing cultures of the human target organ for the particular carcinogen, e.g. oesophagus cultures for nitrosamine experiments, bladder cultures for nitrosoaniline experiments, which could be exposed to various chemical/radiation combinations and monitored for any cell alterations. The second approach was to survey cancer mortality statistics and to produce distribution maps showing areas of the country where particular cancers occurred at above average levels. Factors identified in culture as being interactive could then be studied in high and low incidence counties for the particular cancer.

METHODS

Explant cultures of human organs were set up as follows: Tissue (2cm^2) was obtained from theatre. A small sample of tissue was fixed for histopathological examination to confirm abscence of overt abnormalities and the remainder placed in complete growth medium and rushed to the tissue culture laboratory. The tissue was diced into 2-3 mm^2 pieces and digested for 30 min in 10 ml Earle's Balanced Salt Solution (ESBB) containing 0.1% W/V trypsin and 1 mg/ml Collagenase Type IV. The partially digested pieces were then plated in 25 cm^2 plastic flasks in 2 ml growth medium. Flasks were placed in a 5% CO_2 humidified incubator set at 37° C until required. Medium changes were never given during the culture period.

For carcinogen experiments, appropriate concentrations of carcinogens were added to existing culture medium in a volume of 0.05 ml of growth medium. Where the carcinogen had to be dissolved in a solvent (DMSO, ethanol), a concentrated stock solution was prepared and a dilution of at least 1:100 was made with growth medium prior to addition to cells. Carcinogens were added one day after the explants were set up, allowing time for the explants to

adhere to the plastic, and 12 h prior to irradiation. Irradiation took place using a Cobalt 60 Teletherapy unit delivering 94 cGy/min at 60 (cms) SSD.

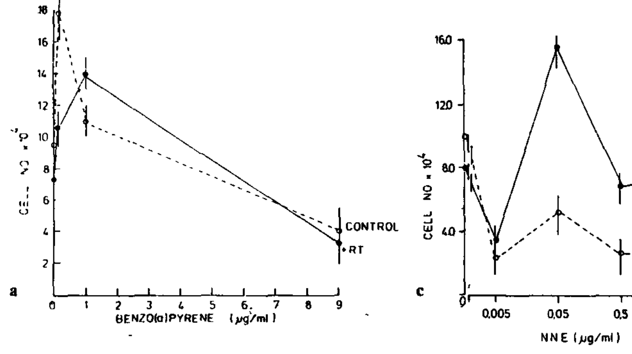
For routine growth estimations cultures were fixed in formalin two weeks after carcinogen and radiation treatment. They were stained with Haematoxylin and Eosin and the area of outgrowth calculated and converted to cell number. Cell proliferation was studied in selected cultures using the monoclonal antibody Ki67 (DAKO) and an indirect peroxidase immunocytochemical development technique.

The epithelial nature of the outgrowth was confirmed in representative samples of cultures using a low molecular weight general cytokeratin antibody with indirect peroxidase development. The presence of stromal or endothelial elements was monitored using anti vimentin and anti human endothelium.

RESULTS

Tissue Culture

Figure 1 shows the effect of increasing levels of three different environmental carcinogens known to affect the oesophagus on the outgrowth of oesophageal explants, assessed at two weeks after treatment. The figures also show the combined effect of the carcinogens and a single dose of radiation (5 Gy).



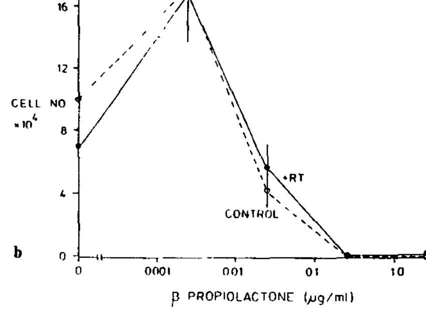


Fig. 1 a–c. The effect of increasing concentrations of benzo(a)pyrene (a), β-propiolactone (b) or n-nitrosoethanolamine (NNE) (c) on the number of cells detected in the outgrowth two weeks after explantation of normal oesophageal epithelium. RT = cultures exposed to 5 Gy gamma rays two days after plating

It is apparent that low levels of benzo(a)pyrene or -propiolactone alone or in combination with radiation stimulate growth. Higher levels of carcinogen inhibit growth. With n-nitrosoethanolamine the carcinogen inhibits growth when used alone, but there is a remarkable growth enhancement when a low level (0.05 g/ml) is used in combination with 5 Gy gamma rays. The percentage increase or decrease in growth for each concentration of carcinogen with and without radiation is shown in Table 1.

	Carcinogen concentration µg/ml	No irradiation		+5 Gy	
		Cell No.	% survival	Cell No.	% survival
β-propiolactone	0	1×10^5	100	7×10^4	100
	0.0025	1.7×10^5	170	1.7×10^5	243
	0.025	4×10^4	40	5.6×10^4	75.7
	0.25	0	0	0	0
	2.5	0	0	0	0
N-nitrosoethanolamine	0	1×10^5	100	8×10^4	100
	0.005	2.4×10^4	24	3.5×10^4	43.7
	0.05	5.2×10^4	52	1.55×10^5	194
	0.5	2.7×10^4	27	6.9×10^4	86.2
	5.0	2.5×10^4	25	7.8×10^4	97.5
Benzo(a)pyrene	0	9.5×10^4	100	7.3×10^4	100
	0.1	1.78×10^5	187	1.06×10^5	145
	1.0	1.1×10^5	115	1.4×10^5	192
	10.0	4×10^4	42	3.1×10^4	42

Table 1

Actual Cell numbers and percentage survival for normal oesophageal mucosal cells exposed K carcinogens and radiation singly or in combination.

Since the combination of radiation and nitrosamine seemed important, it was decided to vary the radiation dose with constant levels of the other two carcinogens and see if a combination leading to dramatic growth enhancement similar to that seen with nitrosamines could be detected for the other carcinogens. Results are shown in Fig 2 for 1 ug/ml benzo(a)pyrene (2a) and for 0.025 ug/ml B-propiolactone (2b). It is apparent that 0.025 g/ml B-propiolactone leads to enhancement of growth at a radiation dose of 2.5 Gy while it had no effect at 5.0 or 7.5 Gy and reduced growth at 10 Gy. With 1 g/ml benzo(a)pyrene, considerable growth enhancement was also seen after a radiation dose of 2.5 Gy but with higher doses, 5-12.5 Gy, a gradual reduction in growth was observed.

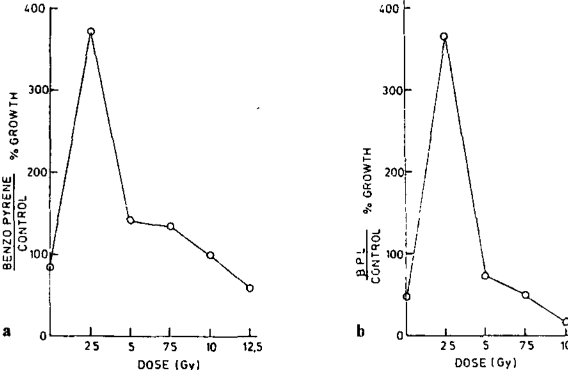


Fig. 2a and b. The effect of increasing doses of Cobalt 60 gamma rays on the number of cells detected in the outgrowth two weeks after explantation of normal oesophageal epithelium. Cultures were exposed to 1.0 µg/ml benzo(a)pyrene (**a**) or 0.025 µg/ml β-propiolactone (**b**) two days after plating and 12 h before irradiation

Where cultures treated with growth stimulating levels of nitrosoethanolamine ±radiation and stained for proliferation using Ki 67 antibody, there was considerable proliferative activity around the edge of the explant and in small pockets throughout the cultures treated with nitrosamine and radiation. Similar results were observed for cultures treated with benzo(a)pyrene or B-propiolactone. The proliferative activity around the outgrowth edge was seen in control cultures although from the area and cell number measurements it was obviously greater in the carcinogen-treated group. The proliferating foci were only seen in cultures treated with carcinogen or carcinogen and radiation and were abundant in the latter group. Since the cells looked different to the epithelial cells and grew over the epithelial cell layer, they were tested for the presence of vimentin and for the endothelial antigen using in situ immunocytochemistry. The cells stained negative for vimentin, excluding the possibility that they were fibroblasts, but were positive for endothelial antigen. They also had characteristic ultrastructural appearance of squamous carcinoma cells. Their nature has therefore still to be resolved.

Quantification of the numbers of foci with respect to dose was performed for both urothelial and oesphageal cultures, the results of urothelium are shown in Fig 3.

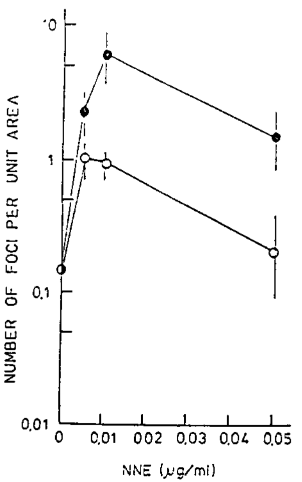


Fig. 3 Numbers of piled upfoci which were K167 positive detected per unit area of outgrowth from human urothelial explants, two weeks after exposure to increasing concentrations of nitrosamines with (o) and without (o) concomitant exposure to 5 gy gamma rays.

It is clear that there is a direct correlation between dose and focus number in the range 2.5 - 5.0 Gy. The combination of nitrosamine with radiation greatly increased both the number and size of the foci. On ultrastructural examination it can be seen that the damage carried by these proliferating focal cells is immense and involves almost total loss of normal mitochondria. Since this suggested that these cells might have defective respiration, experiments were performed where inhibitors of aerobic or anaerobic respiration were used. The results showed that the cells have defective aerobic respiration but quite adequate anaerobic glycolysis. Growth of other cells in the culture, which were not part of the focus, was inhibited by both aerobic and anaerobic inhibitors as were control cells.

The results of experiments where explants were irradiated and numbers of endothelial cells present in the outgrowth after two weeks were counted show that both the absolute numbers of endothelial cells and their frequency relative to other cell types in the explant increased after irradiation. The degree of differentiation (organisation into capillary-like tubes) also showed a characteristic relationship with dose. Differentiation was maximal after explant doses in the region of 2.5-5.0 Gy. Nitrosamine (0.05 mg/ml) increased the numbers of endothelial cells and at radiation doses below 5 Gy had a dose multiplying effect of 1.9. The effect of nitrosamine and radiation 5 Gy on the organisation and differentiation of endothelial cells into capillary tubes was pronounced and appeared to be mediated by the epithelial cells in the culture.

EPIDEMIOLOGY

Maps and tables have been produced showing cancer distributions and assessment of the significance of the patterns is at present underway. The Atlas of Cancer Mortality is in press with the Irish Department of Health.

DISCUSSION

The results presented indicate that low doses of carcinogens combined with low (<5 Gy) doses of radiation can stimulate the outgrowth of cells from oesophageal and urothelial tissue explants and cause proliferation of endothelial cells. All the chemicals are known or suspected carcinogens for oesophageal mucosa and for urothelium in vivo. It is, therefore, of considerable interest of find stimulation of cell proliferation in vitro. Each carcinogen shows a different pattern of dose response and growth peaks at a different combination of treatments. In the case were nitrosamine and radiation were used in combination it is difficult to say whether the nitrosamine is acting by protecting the cells against radiation damage or by enhancing the growth rate of survivors but the immunocytochemical evidence would suggest that proliferation of at least certain groups of cells is actively stimulated by the presence of nitrosamine during irradiation The possibility that radiation is in some way 'metabolically activating' nitrosamine cannot be discounted.

The interaction between chemical carcinogens and radiation in animals has been reviewed by Streffer and Muller, who found mainly additive or subadditive effects with alkylating agents such as nitrosamines. Synergistic effects were sometimes seen but were generally confined to radioresistant organs and to very specific combinations of doses and times of administration of agents. DiPaolo found using cell cultures that the cell killing effect of radiation and the chemical carcinogen tended to mask the transforming effect, but using benzo(a)pyrene treated cells was able to demonstrate considerable enhancement of transformation by exposing cells 48 h later to 1.5-2.5 Gy. Using an experimental lung tumour mouse model, other workers found synergistic effects if fractionated radiation and urethan were combined but not if acute radiation was used.

The finding that proliferation of endothelial cells occurs only in carcinogen±radiation treated cultures is very interesting since angiogenesis is an key factor in epithelial tumour growth. It is interesting to speculate whether the carcinogens themselves stimulate growth of the endothelial cells or whether they stimulate the epithelial

cells to secrete an angiogenesis factor. Preliminary results suggest the latter is occuring.

In all these studies it is apparent that the balance between cell proliferation and cell killing is fundamental to the observation of synergistic interactions. Situations or conditions which show reduced toxicity show synergistic effects, while toxic effects are associated with additive or subadditive effects.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

V. Publications:

Mothersill, C. and Seymour, C.B. (1986). Effect of lactate on the recovery of CHO-K1 cells from gamma radiation damage. Acta Radiol. Oncol., 25, Fasc.1, 71-6.

Mothersill, C., Moriarty, M.J. and Seymour, C.B. (1986). Radiobiologic response of CHO-K1 cells treated with Vitamin A. Acta Radiol. Oncol., 25, Fasc. 4-6, 275-80.

Seymour, C.B., Mothersill, Carmel, Moriarty, M.J. and Tipton, K.F. (1987). The effect of ethanol on the radiation response of CHO-K1 cells. Brit. J. Radiol., 60, 577-81.

Seymour, C.B., and Mothersill, C. (1987). The effect of glycolysis inhibitors on the recovery of CHO-K1 cells from split-dose irradiation. Acta Oncol., 26, Fasc.5, 367-71.

Seymour, C.B., Mothersill, C., Cusack, A. and Hennessy, T.P. (1988). The effect of radiation on the growth of normal and malignant human oesophageal explant cultures pretreated with bleomycin. Brit. J. Radiol., 61, 383-87.

Seymour, C.B. and Mothersill, C. (1988). The effect of glycolysis inhibitors on the radiation response of CHO-K1 cells. Radiat. & Environ. Biophys., 27, 49-57.

Mothersill, Carmel, Seymour, C.B., Cusack, Anne, O'Brien, A. and Butler, M. (1989). Favourable differential sensitivities of normal and tumour bladder explant cultures to combined treatment with radiation and platinum analogues. Acta Oncol. (in press).

Mothersill, Carmel, Cusack, Anne and Seymour, C.B. (1989). Enhanced proliferation of cells from human tissue explants following irradiation in the presence of environmental carcinogens. Rad.Environ.Biophys. 28, 203-212.

Mothersill, C., Seymour, C.B. and Moriarty, M. (1989). Development of radiation transformation systems for epithelial cells - problems and perspectives. Chap. 37 in: Molecular Biology and Cell Transformation, Part 8 of 'Low Dose Radiation' K.F.Baverstock & J.W.Stather, eds). London, New York, Philadelphia: Taylor & Francis, pp.414-422.

Mothersill, Carmel and Seymour, Colin (1989). A new approach to the study of radiation induced epithelial cell transformation. In: 'Cell Transformation and Radiation-induced Cancer'. Edited by: K.H.Chadwick, C.Seymour and B.Barnhart. Bristol & New York: Adam Hilger, pp.91-99.

ABSTRACTS/PRESENTATIONS TO MEETINGS

Mothersill, Carmel, Seymour, C.B.,Cusack, A., O'Brien, A., Moriarty, M. and Hennessy, T.P. Differential response of human normal and tumour tissues in culture to radiation in combination with cytotoxic agents. Meeting of Cell, Tissue and Organ Culture Society, Athens, September 1987.

O'Brien, A., Seymour, C., Mothersill, C., Cusack, A., Moriarty, M. and Butler, M.R. Favourable differential urothelial responses to cytotoxic agents: An in vitro study. British Association of Urological Surgeons, April 1987.

Seymour, C.B. and Mothersill, Carmel. Development of a model system for the study of organ specific toxicity and carcinogenicity using human cells. Joint ESACT-OHOLO Meeting on Modern Approaches to Animal Cell Technology, Tiberius, Israel, April 1987.

Mothersill, C, Seymour, C.B., Cusack, A., O'Brien, A., Moriarty, M. and Hennessy, T.P. Differential response of human tumour and normal tissues in culture to radiation in combination with cytotoxic agents. Proc. 8th International Congress of Radiation Research, Edinburgh, 1987.

McDonnell, M., Mothersill, C., Seymour, C.B., Cusack, A., Moriarty, M. and Hennessy, T.P. (1988). Effect of Novanthrone on the survival of oesophageal explants in culture. Proc. Irish Association for Cancer Research, Galway.

Mothersill, C., Seymour, C.B., Cusack, A., McDonnell, M. and Moriarty, M. (1988). Effect of some carcinogens in combination with radiation on normal human primary bladder and oesophageal cultures. Int. J. Radiat. Biol., 53 (6), 1004.

Mothersill, C., Seymour, C.B. and Moriarty, M. (1988). Development of radiation transformation systems for epithelial cells - Problems and perspectives. 14th L.H.Gray Conference, Oxford.

Mothersill, C., Seymour, C.B., Cusack, A., Hennessy, T.P. and Moriarty, M. (1988). Development of a technique for optimising cancer therapy using a human epithelial model system. Third Scientific Meeting of the British Oncological Association, York.

Mothersill, Carmel and Seymour, Colin (1988). Response of a normal human epithelial culture model to carcinogen exposure. 36th Meeting of European Tissue Culture Society, Gent.

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Mothersill, C., Cusack, A., McDonnell, M. and Seymour, C.B. In vitro predictive testing of tumour response. International Congress of Radiology, Paris.

Mothersill, Carmel, Seymour, C.B., Moriarty, M. and Bonnar, J. A technique for the evaluation of the potential response of human gynaecological tissues to single and combined cancer therapy. Radiation Research Society Meeting, Seattle, U.S.A.

Mothersill, C. and Seymour, C.B. Why can't we transform epithelial cells with radiation? Proc. CEC/DOE/NEB Workshop on Cell Transformation Systems Relevant to Radiation Induced Cancer in Man, Dublin.

BOOK CHAPTERS/INVITED REVIEWS

Seymour, C.B. and Mothersill, C. (1986). Radiation induced transformation in Primary differentiated thyroid cultures. In: `Radiation Carcinogenesis and DNA Alterations'. Ed: F.J.Burns, A.C.Upton & G.Silini. Plenum Press. pp.209-16

PAPERS IN PRESS

Mothersill, C., Cusack, A., Seymour, C., O'Brien, A and Butler, M. (1990). Platinum analogues/Bladder mucosa. Acta Oncol. (in press)

Mothersill, C., Seymour, C.B. and Bonnar, J. (1990). The effect of radiation and other cytotoxic agents on the growth of cells cultured from normal and tumour tissues from the female genital tract. Gynaec. Oncol. (in press)

Mothersill, C., O'Brien, A. and Seymour, C.B. (1990) The effect of radiation in combination with carcinogens on the growth of normal urothelium in explant culture Rad.Environ.Biophys. (in press)

REPORTS AND THESES

Seymour, C.B., Herity, B and Moriarty, M.J., (1990). Atlas of Cancer Mortality in press with Irish Dept. of Health.

Cusack (1990). Effect of Nitrosamines in combination with radiation on the growth of Human Oesophageal mucosa in culture. PhD Thesis University of Dublin.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-094-B

Centre d'Etude de l'Energie
Nucléaire, CEN/SCK
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Head(s) of research team(s) [name(s) and address(es)]:

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Department of Radiobiology
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Boeretang 200
B-2400 Mol

Telephone number: 014-31.18.01

Title of the research contract:

Relation between decorporation of osteotropic alpha-emitters and long term prevention of radiation harm.

List of projects:

1. Incidence of osteosarcoma in mice in dependence of the level of incorporated radionuclides in bone as influenced by decorporation treatment.

Title of the project no.: 1

Incidence of osteosarcoma in mice in dependence of the level of incorporated radionuclides in bone as influenced by decorporation treatment.

Head(s) of project: O. Vanderborght

Scientific staff: G. Schoeters

I. Objectives of the project:

Research sponsored by the former CEC contract BIO-D-381B showed that a 50% decrease of the 226-Ra content due to chronic decorporation with Na-alginate did not alter significantly the bone tumour incidence in mice. The present project was developed to investigate whether the lack of long-term protection was linked to the characteristics of the radioisotope and whether a similar lack of protection was observed after decorporation of other high risk isotopes such as 241-Am (chosen for its predominant role in aged nuclear waste). The decorporative treatment schedules used (Na-alginate after 226-Ra injection and Zn-DTPA after 241-Am injection) were both efficient in reducing the skeletal burden. We investigated whether the radiation dose to sites at risk (bone surfaces) was modified.

II. Objectives for the reporting period:

(1) Evaluation of the efficacity of protracted Zn-DTPA injections to reduce long-term radiation effects. Zn-DTPA therapy started 4 days after 241-Am injection to mimic time elapsing between an accident and start of the therapy. 944 C57BL mice were included in the study.
(2) In addition to the comparison of the efficacity of the decorporative treatments, relative toxicity ratios from 241-Am versus 226-Ra could be calculated with respect to mortality rate and rate of death with bone tumor. This information is important since very few experiments allow in the same species direct comparison of the effects of an actinide with the effects of 226-Ra, the latter are assumed to be the link between animal experiments with bone-seekers and extrapolation of the effects to man.
(3)A. The effect of Na-alginate treatment on 226-Ra distribution in femurs and vertebrae was evaluated via alpha-track autoradiography.
(3)B. The ability of Zn-DTPA treatment to remove a skeletal 241-Am burden was studied via implantation of Am contaminated bones in non-radioactive recipient mice which were subsequently treated with Zn-DTPA.

III. Progress achieved:

1. Methodology

(1) C57BL mice were injected with either 22, 58, 190, 373 or 1197 kBq 241-Am per kg mouse, or 940 kBq 226-Ra per kg mouse or remained uncontaminated. 4 days after injection of 58 and 373 kBq 241-Am/kg mouse, half of the group received intraperitoneal Zn-DTPA injections (50 µmole/kg mouse) once a week during 8 successive weeks. The other mice of these groups were sham-injected with 0.9% NaCl. Also the non-contaminated group was split, one third received Zn-DTPA injections, one third received sham injections and one third was not injected at all. At death, the carcasses were radiographed, suspect tissues were dissected and the tissues were processed for histopathology. Life table analyses was used to compare mortality rate among the groups, also mortality from specific diseases (bone-, liver- and lung tumors, thymic lymphosarcomas, non-thymic lymphoreticular sarcomas and non-neoplastic late-degenerative changes) was compared among the groups via Peto statistics and the non-parametric Breslow modified Wilcoxon test as developed by Hoel and Walburg. To evaluate the efficacity of the treatment schedule for the removal of 241-Am, the 241-Am burden of femurs, vertebrae and livers was radiochemically measured in 8 mice of the Zn-DTPA and corresponding sham-treated 241-Am injected groups at the end of the decorporative Zn-DTPA treatment.

(2) The toxicity ratio of 241-Am versus 226-Ra was calculated with respect to mortality rate and rate of death from bone tumour. The proportional hazards regression model was used and calculations were done in function of injected radioactivity, in function of the average skeletal dose and in function of the average dose rate to the skeleton.

(3)A. C57BL mice were injected with 24 kBq 226-Ra, 4 days later half of the mice received daily 5% Na-alginate in their diet over a period of 6 months. The mice were then killed, femora and vertebrae were removed for examination via alpha-track autoradiography. The tissues were fixed, embedded in Spurr's resin, sections were collected onto CR-39 plastic slides and exposed in a cycled vacuum during 4 weeks. After exposure the slides were etched to develop the 226-Ra alpha-tracks originating from the bone section (NRPB, Chilton, Priest et al., Human Toxicology, 1983).

(3)B. To test whether Zn-DTPA removes any 241-Am from bone surfaces, 241-Am contaminated bones were implanted in not-radioactive recipient mice. The implanted bones had a 1 day old or a 28 day old 241-Am contamination. Previous to implantation in cell-tight diffusion chambers, bone and bone marrow cells were killed by repeated freeze-thawing, to prevent also recycling of Am due to active bone remodelling. Half of the recipient mice were chronically treated with Zn-DTPA injections 4 days after implantation. The Zn-DTPA injections were given once a week during 8 successive weeks.
The 241-Am content of the implanted cortical (femur diaphysis) and trabecular bones (femur epiphysis + metaphysis) was radiochemically measured at the end of the Zn-DTPA treatment period.

Results

(1) Chronic injections with Zn-DTPA, once a week, during 8 successive weeks, and starting 4 days after injection of 58 and 373 kBq 241-Am/kg C57BL mouse were an effective protection against long-term radiation damage. At both dose levels of 241-Am, Zn-DTPA treatment decreased significantly the mortality rate. Death from 241-Am induced bone tumours was significantly reduced at the lower dose level (58 kBq 241-Am/kg mouse). A sparing effect due to Zn-DTPA treatment was also observed for cancers which occur spontaneously in the C57BL mice, but which were enhanced by 241-Am injection (liver tumours, lymphoreticular sarcomas and lymphosarcomas). No tumours did show up in sham-treated mice after 373 kBq 241-Am/kg mouse due to the 241-Am induced shortened life-span. In the corresponding Zn-DTPA treated mice, bone tumors, and a few other malignant tumors were observed. This may be related to the increased life-span of the animals due to Zn-DTPA treatment.
The applied Zn-DTPA treatment was efficient for the reduction of the 241-Am burden with 45 ± 8% in the vertebrae, 40 ± 11% in the femurs and 97 ± 7% in the liver after injection of 58 kBq Am/kg. A similar fraction of the Am burden was removed due to Zn-DTPA treatment in the high dose group. The reduced 241-Am body burden obtained via Zn-DTPA therapy protected the cells at risk for long-term radiation damage. This was in contrast with the lack of protection against long-term radiation damage after a delayed Na-alginate treatment following 226-Ra injection.

(2) The data on survival time and death with bone tumor could be compared between 241-Am injected mice and 226-Ra injected mice of the same C57BL strain (previous experiments). This enabled to compare directly in the same strain the effects of the bone-surface seeker 241-Am to the effects of the bone volume seeker 226-Ra. The proportional hazards regression model was applied and the rate of death with bone tumor was 12.9 times higher after 241-Am injection than after 226-Ra injection if the regression covariate was the average dose to the skeleton. The relative risk was 3.5 if regressed to the injected radioactivity. The mortality rate after 241-Am injection was 20 times higher than after 226-Ra injection if regressed to average dose to the skeleton.

(3)A. Daily treatment with Na-alginate of mice injected with 226-Ra resulted after 6 months in a significantly decreased 226-Ra concentration per g bone ash in the lumbar vetebrae of treated mice ($P < 0.05$). The alpha-track autoradiographs showed no evidence of translocation or redistribution of 226-Ra due to chronic Na-alginate treatment. It is thus unlikely that due to mobilization of 226-Ra from the bones by alginate, a redistribution occurred so that more target cells close to bone surfaces are exposed despite the overall reduced skeletal 226-Ra burden.

(3)B. In an experiment in which translocation of 241-Am from liver to bone and active bone remodelling was prevented, chronic Zn-DTPA treatment (injections once per week during 8 weeks) was not succesful in removing any 241-Am from trabecular or cortical bone surfaces. Zn-DTPA did not remove any significant amount of 241-Am from bones with a one day old 241-Am contamination, or from bones with a 4 weeks old 241-Am contamination.

Conclusion

A delayed chronic Zn-DTPA treatment of mice after 241-Am injection removed a substantial proportion of 241-Am from skeleton and from the liver. This resulted in a protection of the animals against long-term radiation damage (prolonged survival, decrease in tumor incidence). The reduced 241-Am burden obtained with a Zn-DTPA treatment which started only 4 days after 241-Am injection and which lasted 8 weeks protected the cells at risk.
Our transplantation experiment with Am contaminated bones suggested that Zn-DTPA reduced the dose to the cells at risk by preventing redeposition on bone surfaces of 241-Am in the circulation and not by removing the 241-Am which was already bound to bone. We have shown that the additional radiation dose from 241-Am, translocated to bone from the liver and the remodelling bone surfaces, is important for development of late radiation effects.
These results are in contrast with previous findings where a chronic administration of Na-alginate to 226-Ra injected mice in spite of a reduced 226-Ra burden, failed to protect the mice against 226-Ra induced life span shortening and bone tumor induction.
Since alpha-track autoradiography showed no evidence after alginate therapy for redistribution of 226-Ra in the vertebrae e.g. closer to the cells at risk, we assume that the initial 226-Ra radiation dose to cells at risk determines the late radiation effects. Therefore the time lapse between decorporative treatment and 226-Ra radiocontamination should be as short as possible.

The toxicity ratio of 241-Am versus 226-Ra related to average skeletal dose was 20.4 for reduction of life span, and 12.9 for rate of death from bone tumour. This latter value was more than 2 times higher than the risk ratio reported in the literature for beagles and mice. 241-Am did not only induce bone tumors but also increased in a dose dependent way death from other tumors which naturally occurred in the mice (liver tumors, lymphosarcomas, lymphoreticul sarcomas).

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

- SCK-Mol, Belgium - Radioprotection Department, Pathology Section
  Dr. J.R. Maisin
- GSF, Neuherberg, W.-Germany
  Dr. A. Luz
- NRPB, Chilton, U.K.
  Dr. Stather

V. Publications:

Schoeters G.E.R., Vanderborght O. The comparative carcinogenicity of 241-Am versus 226-Ra in various mouse strains. Low dose radiation, ed. K.F. Baverstock & J.W. Stather, Taylor & Francis, London, 1989. Proc. Gray Conference, Oxford, 11-15 September 1988.

Schoeters G.E.R., Vanderborght O. The effect of Zn-DTPA treatment on 241-Am removal from bones implanted in non-radiocontaminated mice. Health Physics 57, 177-180 (1989).

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-185-NL

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Telephone number: 071-276115

Title of the research contract:

Studies on the molecular basis of radiation-induced carcinogenesis.

List of projects:

1. Induction of leukaemia in vivo.
2. Induction of oncogenic transformation in vitro.

STUDIES ON THE MOLECULAR BASIS OF RADIATION-INDUCED CARCINOGENESIS

1. Induction of Leukemia in vivo
2. Induction of oncogenic transformation in vitro.
Contract nr. BI6D-185-NL

Prof.Dr. A.J. van der Eb
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Dr. A.G.Jochemsen
B. KLein

Project 1. Induction of leukemia in vivo

Introduction

The objective of the research proposal, which consisted of 2 projects, was to gain an understanding of the mechanism of radiation-induced carcinogenesis. As a model the induction of leukemia in vivo (project 1) or of oncogenic transformation in vitro (project 2) were chosen. It is generally accepted that induction of cancer is a multistep process, requiring activation of at least 2 complementing oncogenes in the same cell. This complicates studies on the role of physical or chemical agents in carcinogenesis since high doses of the agent are required to activate 2 oncogenes in the same cell. In an attempt to circumvent this problem we have used as starting material normal diploid cells or established cell lines in which one activated oncogene had already been introduced artificially before the radiation treatment. Cells carrying such an activated oncogene would require one step less than the original cells, so that low, more "physiological", doses of radiation could be used to induce subsequent step(s), and the effects of radiation could be more easily studied.

The originally planned approach was to introduce an activated oncogene (ras or myc) into bone marrow cells from an inbred mouse strain, with the use of a retroviral vector. The cells would then be exposed to ionizing- or UV-radiation and transplanted back into (lethally irradiated) syngeneic mice. If these two types of radiation activate different carcinogenic pathways this could result in complementation of different oncogenes. The endpoint is the induction of a form of leukemia.

Results and Discussion

Attempts to introduce genes with the use of retroviral vectors into bone marrow stem cells have met with considerable difficulties. Control experiments carried out in collaboration with the gene-therapy group in the lab (Drs. H. van Ormondt and R. Hoeben) and with Dr. D.Valerio (Radiobiological Institute, Rijswijk) have indicated that it is indeed possible to introduce genes with retroviral vectors into hemopoietic stem cells. However, most of the spleen colonies found after transplantation of the infected bone-marrow cells into lethally-irradiated mice soon lost

expression of the retroviral insert due to inactivation of the LTR-enhancer. Therefore, it was decided to abandon this approach and to try and use bone marrow from transgenic mice carrying an activated oncogene in the germ line. Mouse strains transgenic for the pim-1 oncogene or the c-myc oncogene became available towards the end of 1988 in the laboratory of Dr. A. Berns (Netherlands Cancer Institute, Amsterdam). The oncogenes were regulated by the H-2 or the IgH promoter. Due to lack of sufficient numbers of animals expressing the oncogenes in blood cells it was not possible to carry out the experiments in 1989. In collaboration with Dr. Berns this work will be carried out as soon as sufficient animals can be set aside for the purpose.

Project 2. Induction of transformation in vitro

Introduction

The aim of this study was to introduce an activated oncogene (ras, myc) into primary rodent cell cultures or established rodent cell lines, and to treat the cells subsequently with ionizing- or UV-radiation. It could then be tested which type of radiation treatment can cause a second transformation event resulting in morphological transformation or altered growth behaviour. The method could possibly lead to the development of a suitable and relatively cheap assay of carcinogenic potential of chemical or physical agents.

The results obtained with primary rat embryo or rat kidney cultures indicated that these primary cells are not suitable for the experiments mentioned above, for the following reasons:

1) transfection efficiencies were relatively low, necessitating the isolation of colonies that had been co-transfected with an oncogene and a dominant marker gene conferring neomycin-resistance. This turned out to be a problem because the cells still appeared to have a limited life span and virtually stopped growing before a sufficient number of cells could be obtained to test expression of the cotransfected oncogene. (2) the primary embryo cultures showed high spontaneous "transformation" frequencies.

The established rodent cell lines tested also proved to be unsuitable, because they were all found to be already weakly oncogenic even before transfection of the oncogene. It was decided, therefore, that the best approach would be to focuss on primary cultures derived from mice transgenic for an activated oncogene. Mice transgenic for the pim-1 oncogene were available at the laboratory of Dr. Berns, as mentioned above. The c-myc transgenic mice were not suitable because they expressed the oncogene only in lymphocytes.

Methodology

To minimize the risk of spontaneous transformation, only primary cultures of baby mouse kidney cells were used. 7-8 days old transgenic mice expressing the pim-1 oncogene from the H2 promoter were sacrificed and the kidneys were trypsinized and brought in culture. 2 days after seeding, the cultures were 60-70% confluent and were then irradiated with UV-light or X-rays. (5-10 $J.m^{-2}$ and 300-600 rad, respectively). The cultures were maintained for 6 weeks by changing the medium twice weekly. Cultures from each category were also trypsinized 1 day after irradiation and reseeded at lower cell density or suspended in soft agar medium to score for anchorage-independent growth. The dishes were regularly checked for appearance of morphologically transformed foci or agar colonies.

Results and discussion

Cultures of baby mouse kidney cells from mice transgenic for the *pim*-1 oncogene were irradiated as indicated above. In none of the cultures morphological transformation could clearly be observed, nor was any growth in soft agar detected. This negative result was later explained by the fact that the *pim*-1 gene was only expressed at very low levels in the kidneys of these mice, whereas *pim*-1 protein could not be detected. The experiments will be repeated with a transgenic mouse strain showing high expession of the *pim*-1 oncogene in the kidneys. Of this particular mouse strain only few individuals were available. Therefore, larger numbers of mice have to be bred before the experiments can be carried out. Attempts will also be made to obtain other strains of transgenic mice harboring appropriate oncogenes that are expressed in suitable tissues (preferably baby mouse kidney, alternatively whole embryo).

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B. Klein, A. Pastink, H. Odijk, A. Westerveld and A.J. van der Eb. Transformation and immortalization of diploid Xeroderma pigmentosum fibroblasts. submitted.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-097-UK

Welsh National School of Medicine
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Title of the research contract:

Studies of the mechanism and prevention of low dose radiation carcinogenesis of the thyroid.

List of projects:

1. Studies of the mechanism and prevention of low dose radiation carcinogenesis of the thyroid.

Title of the project no.: Radiation Protection Programme 1985 1989 : Final Report - B16-D-097-UK

STUDIES OF THE MECHANISM AND PREVENTION OF LOW DOSE RADIATION CARCINOGENESIS OF THE THYROID

Head(s) of project:

Professor E D Williams

Scientific staff:

Dr V Wynford-Thomas : Post-Doctorate Research Officer
Miss K Horler : Research Officer
Dr N Williams : Clinical Lecturer

I. Objectives of the project:

To study the quantitiative relationship between low dose radiation and the development of thyroid tumours in the rat and to study the role of the growth stimulatory effect of TSH on tumour development and the value of TSH suppression in the prevention of radiation induced thyroid tumours.

II. Objectives for the reporting period:

To study the relationship between low dose radiation of the thyroid and the number of tumours induced in the goitrogen model, and to study the effect of temporary or permanent interruption of the growth stimulus on carcinogenesis.

III. Progress achieved:

1. Methodology

The study has used radiation to the thyroid from an external source and from ^{131}I followed by longterm goitrogen treatment to give rise to thyroid tumours. The tumours induced were quantified by sub-serial section of the gland, counting the lesions in each section, classifying them as focal hyperplasias or adenomas and measuring the largest diameter of each lesion. Studies carried out included:

(a) studying the effect of very low dose external radiation,

(b) giving ^{131}I followed by antithyroid treatment for varying periods, then killing the animals,

(c) giving ^{131}I followed by antithyroid treatment for varying periods then maintaining animals off antithyroid treatment and killing all groups together,

(d) giving radiation to mice followed by longterm goitrogen treatment followed by a period of remission; and linking the changes to the clonality of the lesions and

(e) giving radiation to one group of animals after they were given a limited course of antithyroid treatment and comparing the number and type of lesions with a group of equal age which had not had previous treatment.

2. Results

A. The results of the (1, 10, 100 rads) were inconclusive - an initial study suggesting a clear dose effect at the lowest rad dose rate could not be confirmed by a second larger study.

B. The results of a study of the rate of onset of focal hyperplasias (FH) and of adenomas (A) after a single dose of radiation followed by prolonged goitrogen therapy were of considerable interest. This study showed that the FH numbers increased lineally with time after radiation but that A numbers initially increased more slowly and later increased more rapidly. The size of the lesion showed a difference between FH and A. The FH size profile remained almost unchanged with time, while the A profile showed a higher mode than FH and a much greater increase in higher diameter lesions with time. Both the changes

in the absolute numbers and in the diameter profile are consistent with a progression of FH to adenoma in the genesis of thyroid neoplasia.

C. The result of a study of the regression of number and size of radiation induced lesions on withdrawal of goitrogen stimulation showed that virtually all lesions produced by 47 or 120 day goitrogen treatment were undetectable by the end of the experiment, while over half of the lesions produced by 200 day goitrogen treatment were present at the end of the experiment, although they were of much smaller size. These results suggest that later lesions are more permanent than the earlier lesions.

D. A study of mice given ^{131}I followed by longterm goitrogen showed that by 46 weeks of treatment a high incidence of adenomas and a significant incidence of adenomas had been achieved. When goitrogen was withdrawn for a period of 4 weeks there was a small drop in the number of adenomas, and a significant drop in the number of carcinomas. Both showed histological evidence of regression. Parallel studies of the clonal origins of the lesions using G6PD histochemistry in mice heterozygous for a G6PD deficiency showed that the lesions were all monoclonal. This study shows that regression does not imply that the lesions were not monoclonal. It suggests that in the modal using radiation followed by longterm goitrogen therapy the tumours induced remained TSH dependent - and their frequency may result from the lack of the requirement to develop TSH autonomous growth.

E. This study is still under evaluation.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

V. Publications:

1(a) Reversibility of the malignant phenotype in monoclonal thyroid tumours in the mouse. (Submitted to B J Cancer).

(b) The rate of onset of neoplastic lesions of the rat thyroid after radiation and longterm goitrogen treatment. Katheryn Horler and E D Williams, in preparation.

(c) The regression of neoplastic lesions of the rat thyroid after radiation and longterm goitrogen treatment. Katheryn Horler and E D Williams, in preparation.

2. Katheryn Horler MPhil - Radiation and Thyroid Carcinogenesis. 1989.

RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-212-NL

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Title of the research contract:

Flow cytometric analysis, computer aided morphometry and histopathology of radiation-induced rat mammary neoplasms as parameters for their biological behaviour.

List of projects:

1. Flow cytometric analysis, computer aided morphometry and histopathology of radiation-induced rat mammary neoplasms as parameters for their biological behaviour.

Title of the project no.:

Flow cytometric analysis, computer aided morphometry and histopathology of radiation-induced rat mammary neoplasma as parameters for their biological behaviour

Head(s) of project:

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Scientific staff:

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Ir. M.J.J. Gijbels
Dr. J.J. Broerse
Dr. J.M.W. Visser

I. Objectives of the project:

Rat mammary tumor models are frequently used for research on the mechanism of human mammary carcinogenesis. However, the value of these models has been criticized as rat mammary carcinomas are clinically less malignant than the human counter parts.
The present study was undertaken in order to study the nuclear DNA content in formalin fixed rat mammary tumors using flow cytometry and to compare these data with histological and cytological characteristics. The applicability of the rat as a model for human mammary gland carcinogenesis will be endorsed if in this respect a similarity between human and rat tumor characteristics can be established.

II. Objectives for the reporting period:

To assess cytological characteristics, such as nuclear area, perimeter and nuclear irregularity, of benign and malignant rat mammary tumors by applying morphometrical methods to archival formalin fixed tumor material as used for DNA flow cytometry and histology.
To evaluate the data on DNA flow cytometry and nuclear characteristics as collected during the whole contractual period and to correlate them with histological signs of malignancy such as cellular pleomorphism, mitotic rate, invasiveness and metastatic capacity.

III. Progress achieved:

1. Methodology

- **Animals.** Paraffin blocks from rat mammary tissues used for FCM were derived from radiation carcinogenesis studies described by van Zwieten (1). In these studies inbred female Wag/Rij rats and Sprague-Dawley rats in the 6th generation of inbreeding were obtained from the specified pathogen free stock colony. After bilateral or multilateral total body irradiation, as described earlier (1), at the age of 8 weeks, they were housed in experimental rooms under conventional conditions. The health of the experimental animals was followed closely by bacteriological and serological monotoring. The animals were kept for their entire lifespan, clinically examined weekly and a complete gross necropsy was performed on those found dead or killed moribund. Histological slides were prepared from all organs and tissues.

- **Histology.** The microscopic examination was performed independently by two pathologists on hematoxylin-phloxine-saffron (HPS) stained sections according to the classification of van Zwieten (1). For our investigations representative samples of the following diagnostic categories were selected: tubular adenomas (n=8), papillary cystadenomas (n=8), fibroadenomas (n=10), noninvasive (n=9) and invasive (n=7), tubulopapillary carcinomas, noninvasive (n=9) and invasive (n=9), cribriform comedo carcinomas and metastasizing carcinomas (n=5).

- **Flowcytometric analysis of DNA content.**
a. Cell preparation and staining. Fifty µm sections were prepared from paraffin blocks of the selected benign and malignant rat mammary tumors, cleared of paraffin by two changes of xylene of 10 minutes each and stepwise rehydrated in a series of alcohol 100%, 96%, 70% and 50% to destilled water as described by Hedley (5). Each step took 10 minutes at room temperature and fluids were changed twice. The rehydrated tissue sections were washed twice with PBS and subsequently subjected to enzymatic digestion to prepare a suspension of free nuclei. The tissue sections were incubated for 30 minutes in 3 ml 0.05% protease XXIV (Sigma P8038) at pH 7.3 at 37°C. The digestion was stopped by adding 10 ml cold PBS. After vortexing the suspension was filtered through a 30 µm mesh nylon gauze, centrifuged, washed with PBS, resuspended and passed 2 times through a 27 gauge needle. The final volume was adjusted to about 10^6 nuclei/ml. To provide an internal standard for normal diploid cells, nuclei of formalin fixed renal tissue of the same mammary tumor bearing rat were isolated using the same procedure and overnight labeled with FITC (3 µg/ml in 0.5 M sodium bicarbonate pH 8.0). The nuclei were subsequently rinsed once with distilled water to remove excess stain. Ten minutes before FCM 0.5 ml labeled renal nuclei was mixed with 0.5 ml of tumor nuclei and the ensuing suspension was labeled with PI in a final concentration of 4 µg/ml.

b. Flow cytometric analysis of DNA content. Cellular DNA content was measured in at least 10.000 cells on a modified fluorescence activated cell sorter, RELACS-3 (3-laser Rijswijk Experimental Light Activated Cell Sorter) using an argon laser operating at 488 nm (0.5 W).
Forward light scatter and time-of-flight measurements were conducted to eliminate aggregates and debris from the analysis. Distilled water was used as sheath fluid. For measuring PI fluorescence, RG 620 and KV 550 filters (Schott Glaswerke, Mainz, FRG) were used in front of a S-20 type photomultiplier (PM). For FITC, a 520 - 550 nm band pass filter (Pomfrett, Stamford, CT) in combination with a 530 nm long pass filter (Schott) was used, also with a S-20 type PM. The filters were used in combination with dichroic beamsplitter FT 570 (Zeiss, Oberkochen, FRG).

c. Ploidy assessment. By measuring simultaneously red (PI) and green (FITC) fluorescence a scatter plot resulted, which was used to provide two histograms: one representing renal nuclei (both green and red fluorescence) and one representing tumor nuclei (red fluorescence only) (See progress report 1988). From these histograms the peak channel number of the first major peak of the tumor cell suspension was derived and divided by the peak channel number of normal renal nuclei (DI). To check whether the presence of FITC influenced the fluorescence intensity of PI, peak channel numbers of nuclei of the same renal tissue stained with PI or PI + FITC were compared. It appeared that in the presence of FITC a higher red fluorescence was observed. This factor could vary from 1.00 to 1.14. The amount of the contribution may vary with dye concentration and filter choices. Therefore, a correction factor was determined for each run using the same standard deparaffinized renal tissue. Histograms with a coefficient of variation of less than 8% in the diploid peak were considered of sufficiently good quality for further analysis. Tumors with a G0-G1 population with a DI between 0.9 and 1.1 were defined as diploid. A cell population with a DI ≤ 0.9 was considered to be DNA hypoploid and a cell population with a DI between 1.1 and 1.8 to be DNA hyperploid.

- **Cytological characteristics.**

a. Mitotic activity index. In 24 tumors we tried to relate the mitotic index to DNA aneuploidy and histological signs of malignancy. To this end we chose material from tubulopapillary tumors in various degrees of malignancy i.e. tubulopapillary adenomas, noninvasive and invasive tubulopapillary carcinomas. The number of mitoses was counted in 10 random fields at x400 magnification in the histologically most malignant areas of the tumors.

b. Computer aided morphometry. To this end 3 µm thick slides were prepared of the same 24 tumors selected for establishing the mitotic activity index. Morphometric analysis was performed in the most atypical areas as judged by hypercellularity, nuclear and cellular pleomorphism and relatively high mitotic rate.

Photomicrographs of these areas were made with a 63x objective and printed at a final magnification of 2500x, resulting in nuclear images of at least 20 mm diameter. At least 25 epithelial nuclei with intact nuclear outline were randomly selected for assessment of perimeter, area and formfactor PE (4π area/quadrated perimeter). Nuclear outlines were directly measured by using a cursor and graphic tablet coupled with a MOP Videoplan (Kontron, Munich, FRG; software version 5.42) microcomputer.

- **Statistics.** Differences in frequency distribution in data groups were assessed with the Fisher's Exact Test or Students T test. The level of significance was set at a p of ≤ 0.05.

2. Results

Out of 65 tumors, 43 were DNA-diploid (66%) and 22 aneuploid (34%). Twelve (18%) of the DNA aneuploid tumors were hypoploid. There were no animals with a tetraploid tumor according to the criteria formulated by Rutteman (6). The results of histological typing and of FCM examinations are summarized in Table 1.

Benign tumors. Five (18%) of the 26 benign tumors had aneuploid G0-G1 peaks. Four of the 5 aneuploid tumors were hypoploid.
Within the series of benign tumors there was no relation between histological typing and ploidy status.

Malignant tumors. Seventeen (44%) of the 39 malignant tumors had aneuploid G0-G1 peaks. Eight (47%) of these 17 aneuploid tumors were hypoploid. Nine (47%) rats of the 19 with a tubulopapillary carcinoma had an aneuploid pattern while 8 (40%) of the 20 with a cribriform comedo carcinoma were aneuploid. Therefore, tubulopapillary carcinomas and cribriform comedo carcinomas did not differ in DNA ploidy status.
Within the series of malignant tumors no correlation was observed between degree of malignancy (non-invasive ≤ invasive ≤ metastatic) and percentage of DNA aneuploid tumors (Table 2).
Within the group of benign tumors diploid cases were more frequent than in the group of malignant tumors (82% and 56% respectively) ($p \leq 0.05$).
Within the group of malignant tumors hyperploid cases were more frequent (9/39) than in the group of benign tumors (1/26) ($p \leq 0.05$). There was no difference in hypoploidy between the malignant (8/39) and benign groups (4/26).

DNA and mitotic activity index. In the series of tubulopapillary tumors, tubulopapillary carcinomas had a significantly higher mitotic activity than the tubulopapillary adenomas ($p \leq 0.05$) (Table 3). Mitotic activity index varied considerably between tumors. No difference was observed between invasive and non-invasive carcinomas. There was no correlation between the mitotic activity index and the DI (data not shown).

Table 1 DNA ploidy distribution in 65 rat mammary tumors

	Number of animals	Diploid	Aneuploid total	hypopl.	hyperpl.
Benign					
tubular adenoma	8	7(88)[a]	1(12)	1	0
papillary cystadenoma	8	6(75)	2(25)	1	1
fibroadenoma	10	8(80)	2(20)	2	0
		21(82)	5(18)	4	1
Malignant					
tubulopap.carc.	19	10(53)	9(47)	6	3
noninvasive	9	4(44)	5(56)	2	3
invasive	7	4(57)	3(43)	3	0
metastatic	3	2	1	1	0
cribriform-comedocarc.	20	12(60)	8(40)	2	6
noninvasive	9	3(33)	6(66)	2	4
invasive	9	7(78)	2(22)	0	2
metastatic	2	2	0	-	-
		22(56)	17(44)	8	9

[a]Number of animals, percentages in parentheses.

Table 2 Relationship of histological malignancy grade and ploidy levels

	Number of animals	Diploid	Aneuploid total	[hypopl.	hyperpl.]
Benign	26(100)	21(82)[a]	5(18)	[4(15)	1(4)]
Malignant	39(100)	22(56)	17(44)	[8(21)	9(23)]
noninvasive	18(100)	7(39)	11(61)	[4(22)	7(39)]
invasive	16(100)	11(69)	5(31)	[3(19)	2(12)]
metastatic	5	4	1	[1	0]

[a]Number of animals, percentages in parentheses.

Table 3 Relationship of mitotic activity index and tubular (papillary) tumors

	Number of animals	Mitotic activity index
tubulopap. adenoma	8	9,6 ± 8,6[a]
tubulopap. carc. noninvasive	9	25,1 ± 16,0
tubulopap. carc. invasive	7	28,7 ± 18,3

[a]Mean ± s.e.

Table 4 Mean and standard error (s.e.) of various morphometrically established nuclear features in rat mammary tumors

feature (mean ± s.e.)	1 tubulopapillary adenoma	2 non-invasive tubulopapillary carcinoma	3 invasive tubulopapillary carcinoma	
perimeter	24.0	27.6	28.2	x
s.e.	± 0.73	± 2.3	± 1.6	
area	42.3	58.0	60.1	x
s.e.	± 2.8	10.6	6.7	
formfactor PE	0.911	0.909	0.921	
s.e.	0.078	0.094	0.004	
D MAX	8.0	9.2	9.3	x
s.e.	± 0.23	0.7	0.5	
Number of animals	8	9	7	

[x] Significantly different from value in column 1.

Morphometry. The results of the nuclear measurements performed on histological slides of the 8 tubulopapillary adenomas, 9 non-invasive tubulopapillary carcinomas and 7 invasive tubulopapillary carcinomas are summarized in Table 4. The mean values for area, perimeter and maximal diameter D.Max were greater in both carcinoma groups as compared to the adenomagroup, reaching the level of significance only for the invasive carcinomas as compared to the adenoma category. Overlap between the 3 groups was appreciable.

3. Discussion

The clinical behavior of rat mammary carcinomas is different from that of human breast cancer i.e. they are characterized by a noninvasive or microinvasive growth pattern and a low frequency of metastasis [about 5 % (1)]. Their volume doubling time is lower and may even be less than that of benign rat mammary tumors (7).

In the present study, DNA ploidy patterns were determined in spontaneous and radiation and/or estrogen treatment induced rat mammary tumors. The DNA content of the tumor cells was related to DNA content of diploid reference cells and expressed as the DI i.e., the ratio of the mode of the relative DNA content of the $G_{1/0}$ cells of the sample divided by the mode of the relative DNA content of the diploid $G_{1/0}$ reference cells.

According to the convention on nomenclature for DNA cytometry (8), the reference cells were mixed with the cells of the tumor sample before PI labeling. The reference cells were derived from paraffin blocks of renal tissue of the same rat to assure identical treatment of sample and reference cells before PI staining used for DI estimation. In order to recognize the added reference cells from those of the sample they were prepared and prestained with FITC separately. Subsequently, both sample and FITC labeled cells were mixed and stained with PI. The small shift of PI intensity of the reference cells due to double staining as measured in parallel control experiments using renal nuclei stained with PI only and PI + FITC was corrected for and was never greater than a factor 1.14. Using this method we could clearly recognize the normal reference $G_{1/0}$ peak and therefore distinguish DNA hypoploid and DNA hyperploid peaks. This is impossible when normal cells in the tumor are used as a reference. In the latter situation, the first peak is assumed to represent normal $G_{1/0}$ cells.

In the present study 5 (20%) of 26 benign tumors were DNA hypoploid and 1 (4%) hyperploid. These findings contrast with data obtained in man where benign mammary tumors are characterized by a DI of about 1. However this may be species related as comparable percentages of DNA aneuploidy have been reported in canine benign mammary tumors (6,9). Of the 39 malignant rat tumors 17 (44%) were aneuploid and 9 (23%) of these were hyperploid. Thus DNA aneuploidy and especially DNA hyperploidy was more frequent in malignant than in benign rat mammary tumors. No differences in this respect were observed between invasive and non-invasive carcinomas.

In the present study we observed a lower incidence of aneuploidy (44%) in the rat mammary carcinomas than normally observed in human mammary carcinomas (4,10). Others, however, also observed a relatively low incidence (44%) of aneuploidy in human mammary carcinomas (11).

In man DNA aneuploidy is associated with a poor prognosis (2,12). However, rats with mammary carcinomas do not die because of generalized metastatic disease but in the majority are euthanized because of the large size of the tumors or ulceration of overlying skin. They, therefore, might have lived for a much longer time. In man prognosis is mostly determined as survival after time of diagnosis and treatment. Rats are left untreated and histological diagnosis is made after necropsy.

In our study DNA hyperploidy was observed more often in malignant than in benign tumors. But within the carcinoma category hyperploidy or the degree of DNA hyperploidy was not more frequent in the histologically or clinically more malignant cases as has been reported in man (10).

Baak et al. (2,3) noticed that, in addition to histological appearance, quantitative nuclear parameters and the mitotic rate are good predictors of prognosis in breast cancer. In our study, we indeed found a correlation between histological malignancy, nuclear features and mitotic activity index, however, no differences were observed between invasive and noninvasive malignant tumors.

In conclusion, our study shows that the category of histologically malignant rat mammary tumors differ significantly from benign rat mammary tumors in the relative frequency of DNA hyperploidy, mitotic activity index and some quantitative nuclear characteristics. This lends support to the contention that the histologically malignant rat mammary tumors, although clinically less malignant, are in many aspects comparable to their human counter parts.

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RADIATION PROTECTION PROGRAMME
Final Report

Contractor: Contract no.: BI6-D-099-D

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Telephone number: 56-911661

Title of the research contract:

Late somatic effects of ionizing radiation on the mammalian organism.

List of projects:

1. Promotion of cooperation in the fields of late somatic effects, such as non-stochastic damage, carcinogenesis, effects of incorporated radionuclides and consequences of in utero exposure.

Title of the project no.: 1

Promotion of co-operation in the fields of late somatic effects, such as non-stochastic damage, carcinogenesis, effects of incorporated radionuclides and consequences of in utero exposure.

Head(s) of project:

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Scientific staff:

I. Objectives of the project:

The objective of the European Late Effects Project Group (EULEP) is to plan, promote, execute and analyse co-operative research relevant to the understanding of the biological effects of exposure of living organisms to ionizing radiation.

II. Objectives for the reporting period:

1. To co-ordinate research activities on specific aspects of the late effects of ionizing radiation by means of problem-oriented task groups;
2. To maintain, develop and standardise relevant methodologies, to introduce new techniques, and to initiate appropriate training activities, by means of four committees:
   - Committee of External Radiation Dosimetry and Techniques
   - Committee of Internal Radiation Dosimetry and Techniques
   - Committee of Pathology
   - Committee of Cell and Molecular Biology;
3. To plan regular symposia on aspects of late effects studies.

III. Progress achieved:

During the period covered by this report, EULEP carried out an internal reorganisation by which the co-ordination of research activities is achieved through a number of task groups. Each of these has been established to deal with a specific problem that has been studied by a co-ordinated effort in the member laboratories.

The four standardisation committees referred to in section II have continued to play an important role in the activities of EULEP, and their work will be reported first.

Committee of External Radiation Dosimetry and Techniques

For the purposes of standardisation of experimental conditions, so that results of animal studies are directly comparable between member laboratories, the committee has organised periodic intercomparisons of the dosimetry of X-ray exposures. Mouse phantoms filled with thermoluminescent dosimeters have been mailed to each institute and irradiated under an agreed protocol. The read-out of the TLDs and derivation of the absorbed doses have been performed at the National Institute of Public Health and Environmental Protection (RIVM) at Bilthoven, The Netherlands. The results have been evaluated jointly by RIVM and TNO.

The fifth dosimetry intercomparison was performed in 1983-1985 and the mean results for the different laboratories were within ± 5% of the readings at the reference laboratory for all except one of the eleven participating institutes. That one case was followed up in 1986-87, when it was found that the discrepancy was not due to the dosimetry instrumentation itself, but probably resulted from a change in the position of the monitor chamber. Separate estimates were also made in this intercomparison of the degree of uniformity of dose distribution across the phantom.

A sixth dosimetry intercomparison was planned during 1988 and carried out in 1989. Preparations included various refinements to the procedures at Bilthoven. Fifteen laboratories participated in the first session and the measurements were performed as before. Further estimations were made for those institutes with deviations close to or greater than ± 5%. The evaluation of the results will be completed in early 1990, but preliminary data for the absorbed dose in the centre of the phantom reveal that the figures were in excess of ± 5% of the value

at the reference laboratory for four participating institutes. Concerning the distribution of absorbed dose in the mouse phantom, four participants did not meet the criterion for a uniform dose distribution, viz. a ratio of maximum to minimum doses of less than 1.10. These discrepancies will be followed up by consultations in the first instance.

The EULEP protocol for X-ray dosimetry and exposure arrangements was published in 1985, with an abbreviated version published as a journal paper. This has proved of immense value as a handbook and source of guidance on the subject.

Other activities of the committee have included dosimetric aspects of total-body irradiation of patients for bone marrow transplantation in the treatment of acute leukaemia and other diseases. Symposia dealing with this topic were organised in 1986 and 1988, in conjunction with other bodies representing the clinicians involved.

Committee of Internal Radiation Dosimetry and Techniques

Since its formation in March 1986 the committee has pursued activities in three areas:

(i) Task Groups. The committee has given support to the six task groups which lie within its sphere of responsibility: Lung, Decorporation, Stem Cells, Bone, Fetal Dosimetry and G-I Tract. Another task group, on Early Indicators of Pulmonary Damage, was set up during the reporting period but was later dissolved when two of the three member laboratories had to cease work in this field. Encouragement was given to the task groups on bone, stem cells and fetal dosimetry to hold joint meetings; two such meetings have been held to which eminent guest speakers were invited. These proved very successful with the larger audience leading to lively discussions and considerable cross-fertilisation of ideas.

(ii) Training and Standardisation. Recognising that several EULEP laboratories were working on various aspects of alveolar macrophage function the committee organised a one day workshop on macrophages in 1987. This workshop was partly educational, with critical review papers from world-renowned experts in various aspects of macrophage function, and partly provided a forum for the detailed discussion of the work on macrophages being carried out in the various laboratories. This very successful venture initiated a number of new collaborative projects between member laboratories and led to an extensive programme of worker

exchange in order to standardise the experimental methods being used in the different laboratories. Scientific discussions were held during committee meetings on Some Problems of Beta Dosimetry (1987) and on Cell-Specific Dosimetry (1989).

(iii) Committee Scientific Work. Following the accident at the Chernobyl nuclear power station many EULEP members received urgent requests for assessments of the committed radiation doses likely to be received by particular groups in the population following intake of radionuclides in foodstuffs or in water. Acting on a request from Council a working group of the committee developed a simple computer program for the calculation of the absorbed radiation dose to 21 tissues of adults, and 1 and 10 year-old children, resulting from ingestion of any of the 50 most important radionuclides. This program - DOSELIB - contains dosimetric information from the database of the Federal German Health Office, upgraded to take account of the new metabolic models for the actinides proposed in ICRP Publication 48, and the co-operation of Professor Alexander Kaul and his colleagues is gratefully acknowledged. The program is suitable for any IBM-compatible computer and is available on floppy disc. Plans have been made to update this program to take account of any changes in dose limits in new Recommendations of the ICRP which are currently in preparation.

Co-operation with other organisations - In 1988 a formal link was established with Committee 6 (Assessment of Internal Dose) of the EURADOS-CENDOS European Radiation Dosimetry Group, as a result of this link each committee is now represented by its chairman of meetings of the other. A joint EULEP-EURADOS application, involving five laboratories (4 EURADOS + EULEP), on the Assessment of Internal Dose from Radionuclides using Stable Isotope Tracer Techniques in Man was selected for funding under the European Communities Radiation Protection Programme 1990-91.

Committee of Pathology

The main goals of this committee are (1) to further standardise and update diagnostic terminology as used by pathologists of the EULEP member laboratories and (2) to increase the expertise of members on various morphological aspects of late effect studies in laboratory animals, including the application of new techniques.

The committee has organised yearly slide seminars each covering a different organ system. These are intended to give members an up-to-date

overview of specialist diagnostic procedures as well as providing a forum for discussion of problem areas and standardisation of terminology. Outside experts were invited to lecture on specialist topics in which expertise was lacking within the committee. Slide seminars were held on the respiratory tract, the urinary tract, the haemopoietic tissues, the bone marrow (with particular emphasis on myeloid lesions), and on the neuroendocrine and APUD system.

The committee also organised symposia on more general topics in animal pathology, the subjects of which included Comparative Pathobiology of Experimental in vivo and in vitro Systems, Total-body Irradiation, and Quantitative Morphology. In addition members of the Committee contributed to other EULEP workshops and symposia, including the symposium on effects after combined exposure to ionizing radiation and chemical substances.

The committee has maintained a consultation centre for problem cases. The majority of these were discussed during the annual slide seminars.

Two further fascicles of the EULEP Atlas were published (E Kunze: Preneoplastic and neoplastic lesions of the urinary bladder of the rat; C Zurcher: Neoplastic and non-neoplastic lesions of male and female genital tract of the mouse and rat); two fascicles are in press (P Bannasch and H Zerban: Preneoplastic and neoplastic lesions of the kidney of the rat; P K Pattengale: Neoplastic lesions of the mouse lymphoid system) and a further fascicle (on skin) is in preparation.

The activities of the committee greatly helped in maintaining and upgrading the expertise of individual pathologists in the member laboratories. The decision to invite outside experts to give "state of the art" lectures at the slide seminars has proven very valuable, providing members with an opportunity to discuss the most modern developments in pathology, and to use these to improve diagnostic precision in animal experiments. Maintaining this level of expertise in the committee is essential for the maintenance of the high quality pathology support given in many studies performed in EULEP laboratories.

Committee of Cell and Molecular Biology

Following the establishment of this new committee in 1986, it was proposed that its major activities should consist of (a) individual and collective training courses for research workers with no previous

experience in molecular biology, and (b) workshops designed to introduce experts in the different laboratories to novel techniques which had been developed in one of them.

At two meetings in December 1986 and January 1987 the committee determined the priority to be given to various aspects of training in molecular biology. It was agreed to provide on the one hand a general introduction to the techniques of molecular biology, and on the other hand training on molecular hybridization in situ. The committee also decided which form the training courses should be given in order to be the most profitable.

In May 1987 a training course took place on "The fundamentals of molecular biology"; this was held in the laboratories of CEN, Mol, Belgium, and was entirely funded by EULEP. It was attended by 12 young research workers from EULEP-associated laboratories, coming from 8 different countries. The participants became acquainted with DNA and RNA purification from mammalian tissues, restriction enzyme analysis, transfer of RNA and DNA onto immobilising supports, radioactive labelling of molecular probes, and subsequent molecular hybridization.

In 1987 the committee also supported a 2-month stay of a EULEP-associated member at the University of Brussels, in order to introduce into the EULEP network the technique of in situ molecular hybridization which was much needed.

On several occasions the committee has organised or co-ordinated workshops for the members of the various task groups involved in the use of cell and molecular biology techniques. There were two such workshops in 1987 and two in 1988.

TASK GROUPS: Eleven task groups were established in 1986, some representing new lines of work within EULEP but others broadly continuing work which had been co-ordinated previously. Since that date other task groups have been established to meet changing demands and new opportunities for collaboration. Some of the more significant aspects of progress achieved are as follows:

(a) Molecular Approach to the Study of Radiation-Induced Osteosarcoma

The objective has been to study the pathogenesis of radiation-induced osteosarcoma at the molecular level. A powerful feature of the programme has been the parallel development of studies with cell lines,

organ culture in vitro, and in vivo pathogenesis. The infection of osteoblast-like cell lines with viral sequences from radiation-induced murine osteosarcoma has resulted in neoplastic transformation and abrogation of osteogenic differentiation. Osteosarcoma-like lesions have been produced in organ culture of cartilage in vitro, which are transplantable in mice; this model has been used to study cell migration and invasion.

The participation of retroviruses is the major focus of interest, together with the role of certain oncogenes. Newly integrated proviruses in radiation-induced osteosarcomas have been analysed and cloned. The viruses are highly homologous but the different clones exhibit different pathogenicity in bone, suggesting rather subtle differences among the proviral genomes. The RFB osteoma virus was cloned and its LTR sequence was compared to that of other bone tissue material. Studies on the proviral integration sites in RFB-induced osteomas have been co-ordinated. The study on the localisation of the viral sequences which may confer pathogenicity on the RFB virus has called for a co-ordinated experimental strategy, including the generation of recombinants between RFB and a highly homologous non-pathogenic virus, and pathological studies on transgenic mice.

(b) Molecular Approach to the Study of Radiation-Induced Haemopoietic Neoplasias

Originally this task group was established to study molecular aspects of the induction of thymic lymphoma (TL) in the C57Bl mouse. It had been found that there were new proviral inserts in some primary tumours and in all cell lines grown in vitro or serially transplanted. The possible expression of RNA synthesis from a cellular gene promoted by new integrated proviruses was studied; there was found to be unexpected activation by RadLV of endogenous rat proviral genomes.

A direct test of the Kaplan hypothesis was made, to determine if the induction of TL by radiation might be directly mediated by a leukaemogenic retrovirus. In general, novel provirus representing a recombinant of endogenous proviruses was not found in the affected cells, thereby failing to confirm the hypothesis.

The final conclusion of that collaborative work was that, even if RadLV-like proviruses might facilitate cell growth, they should be no longer considered as inducing agents or as essential factors in the

production of TL. Some work is continuing on TL induction, however, directed towards the interaction between radiation-induced preneoplastic cells and the thymic microenvironment.

In the last year, the composition and aims of the group were changed, to concentrate more on myeloid leukaemia in the CBA mouse. Specific chromosomal lesions have been studied which are commonly found in leukaemic cells, also the possible activation of genes resulting from this which may be concerned with leukaemogenesis itself. Attention has focused chiefly on a region of chromosome 2, where structural rearrangements had suggested an association with enhanced activity of the gene for interleukin-1 beta. Further studies have suggested however that IL-1 beta deregulation is probably not an initiating event in myeloid leukaemia. Studies are continuing on the sites of chromosome 2 rearrangement which show remarkable radiation fragility.

(c) Cell Biology of Haemopoietic Tissues

The task group was established initially to investigate the cell biology of thymic lymphoma, complementing the molecular biology described above (b). These studies used two models for investigating relevant cellular factors, a thymic graft to thymectomised irradiated animals, and the transfer of pre-leukaemic cells and/or factors to irradiated recipients. In situ hybridisation studies have been performed.

The group has been actively looking for the role of lymphokines after irradiation, during leukaemogenesis in vivo and during the long period preceding the establishment of lymphoma lines in tissue culture.

With the shift in emphasis towards myeloid leukaemia noted above, the group is now redirecting some of its efforts to include myeloid as well as lymphoid malignancies, but the overall aim remains to investigate the cellular basis of long-term consequences of irradiation in haemopoietic tissues. In the two cases growth factors seem to play critical but different roles.

(d) Cellular Basis of Late Vascular Changes in the Irradiated Brain

Over many years this project has elucidated important aspects of the pathogenesis of damage to certain regions of the irradiated brain, seen from 12 to 24 months after doses of 20 Gy X-rays. The group has integrated methodological and physiological studies with the aim of delineating and evaluating the areas at risk in the central nervous

system, evaluating the degree of risk and also factors that may modify the expression of the late effects.

It was found that vasodilatation preceded demyelination. There was no early decrease in vascular density or vasodilatation in the deep cortex, but both were evident at 12 months post-irradiation. While the blood-brain barrier did not break down, the antipyrine distribution space increased after 14 - 18 months. There was a decrease in nutritional blood flow.

The white matter of the brain is the more sensitive part, and the fimbria hippocampi (FH) is the most sensitive region. The earliest manifestation of damage has been defined in terms of a "tissue injury unit", consisting of enlargement of endothelial cells and astrocytes, blood vessel dilatation, and blood vessel wall thickening. Electron microscopic studies also showed primordial telangiectatic lesions in the FH. The appearance of the tissue injury unit occurred before the development of damage to the nervous tissue itself, ie, demyelination; moreover, it represents the most sensitive response of the CNS to irradiation.

(e) Non-Stochastic Effects on the Skin

Two task groups have been active on different aspects of the late radiation response of the skin. One has been concerned to evaluate the contributions of epidermal basal cell damage and dermal cell killing to the eventual desquamation and epidermal breakdown, as a function of depth-dose and field size. This is of importance in defining the risk from local irradiation by energetic beta-emitters in contact with the skin. Mouse and pig skin were compared, using sources of ^{90}Sr and ^{170}Tm (thulium) of 1, 5 or 9 mm diameter. In the case of pig skin, the classical pattern of epithelial damage was not seen over the first few weeks: damage occurred rather by endothelial and fibroblast cell death in the dermis, leading to capillary loss from the papillary dermis and epidermal breakdown from 14 days after doses of 200-500 Gy. In the mouse, 300-1500 Gy again caused damage in the dermal as well as epidermal layers, leading to epithelial and follicle breakdown by 6 days.

More recently a second task group on skin began work on the effects of residual radiation injury from an initial exposure on dermal and subcutaneous vascular and connective tissue. The skin was to be re-exposed to high energy beta particles from ^{90}Sr plaques. Two strains of

pig were compared, and different anaesthetic procedures which were in use in the different laboratories. The response to ^{90}Sr irradiation in terms of moist desquamation after 9 weeks was found to be very different for the two strains. Using either anaesthetic (azaperon/etomidat or halothane/N_2O/O_2), the large white pigs showed a lower ED_{50} for moist desquamation than the Gottinger miniature pigs, by a factor of 1.3-1.4. With azaperon/etomidat there were variations in the ED_{50} between different skin fields, but not with halothane/N_2O/O_2 unless the percentage of O_2 in the mixture was reduced from 70% to 21%.

(f) Radiation Effects on the Heart

A collaborative project was established to examine morphological and functional changes in the irradiated rat heart. Wistar and Sprague-Dawley (S-D) rats were used, and doses selected for each (17.5 and 20.0 Gy respectively) which resulted in a comparable latent period from irradiation to death from cardiac failure. Similar pathological lesions were seen in the two strains at 100 days. The first effect was a rapid decrease in capillary density and loss of alkaline phosphatase by capillary endothelial cells, both seen by 28 days and before any marked pathological changes. The correlation with functional changes was however complex. Cardiac output measured in S-D rats decreased by 28 days, whereas in Wistar rats it increased over the same period of time. The percentage of cardiac output distributed to the ventricular muscle, assessed by ^{86}Rb extraction, increased in S-D rats but did not change significantly in Wistar rats. In fact the rate of blood perfusion per gram of venticular muscle calculated from these data did not change greatly in either strain of rat, compared with controls, over the period of observation. Thus the cardiovascular system compensated for the marked morphological changes, though not in the same way in the two strains of rat.

(g) Effects of Radiation on Pre-Implantation Mouse Embryos

The effects of X-rays, neutrons and tritiated arginine and thymidine have been studied on 1- and 2-cell embryos from different mouse strains. In one strain similar to the NMRI mouse, there was an increased teratogenic risk: in particular an increase in the number of gastroschises. The sensitivity was very pronounced at the 1-cell stage, but changed within hours. There was no detectable threshold at the 1-

cell stage, unlike multicellular embryos (32 to 64 cells) where abnormalities appear only above 0.5 - 1.0 Gy. C57Bl mouse embryos did not show increased teratogenic risk at the pre-implantation stage.

Chromosome aberrations have also been studied at this stage of development. Tritiated thymidine did not increase the frequency of aberrations at the first mitosis, but arginine did produce a rise in the second and third divisions, thymidine less so. Dose-effect curves were linear up to 1.88 Gy X-rays and 0.75 Gy neutrons.

It has been shown that zygotes of certain mouse strains are very sensitive to radiation-induced block of the cell cycle at the G2 stage, e.g. after 1 Gy X-rays. This is dependent on the maternal, not the embryonic genotype. Protein synthesis in blocked embryos differed from unirradiated controls by the absence of phosphorylation of some 35 kD proteins at the time of the first cleavage. Caffeine can reverse the G2 block, probably by restoring, directly or indirectly, the post-translation modification of these proteins. Another current aspect of this work is to ascertain whether G2 block involves the nucleus of the 1-cell embryo; the nucleus does not normally control development until the 2-cell stage.

(h) Effects of Radiation on the Development of the Central Nervous System

The aim has been to assess radiation-induced damage to the fetal CNS by means of animal experimental models. A number of related endpoints have been studied, and some experiments have been extended down to low dose levels.

Structural defects in the developing brain have been demonstrated in a number of models. The development of the cerebral cortex was found to be very sensitive to irradiation in the mouse, manifested as disturbed proliferation and migration of neurones and a disturbed branching pattern of neurones in the cortex. Image analysis showed that the degree of neuronal disturbance decreased with increasing age at time of exposure: as little as 0.1 Gy affected the alignment of neurones at 12 days post-conception (p.c.), rising to 0.5 Gy as the minimum dose at 17-18 days p.c.

A part of the corpus callosum has been found to be the most sensitive area to structural damage. Significant atrophy was found in 3-month old rats exposed to only 0.15 Gy X-rays or 0.05 Gy neutrons at 15

days p.c.

Other studies in the NMRI mouse showed that 0.9 Gy X-rays at day 12 p.c. caused cell depletion especially in the proliferating ependymal zone. 1.9 Gy completely prevented the development of this area.

Recent studies have investigated lectin-binding sites on neuroblasts of several regions of the embryonic brain (9-10 days p.c.). The binding of certain lectins has been shown to occur 6-12 hours after doses as low as 0.125 Gy, disappearing again 24-36 hours later; this binding was presumably either absent or masked in the unirradiated, normally developing brain.

A new addition to this collaboration is behavioural studies conducted on mice, using a maze to assess effects of radiation on spatial memory. Performance at 60 days of age is being studied after X-irradiation at different stages of organogenesis.

(i) Interspecies Comparison of Lung Clearance

The purpose of the task group has been to quantitate the kinetics of various particle clearance and retention mechanisms in the lung in different animal species and in man. Special emphasis has been placed on the two main clearance mechanisms for long-term retained aerosol particles: the translocation of dissolved material from the lungs to blood, and "mechanical" particle clearance via the pharynx to the gastro-intestinal tract.

In the last five years the task group has carried out three collaborative interspecies comparisons of lung clearance, and a fourth is in progress:

(i) Lung clearance of inhaled porous monodisperse cobalt oxide particles of 0.8 µm and 1.7 µm diameter, in man, baboon, dog, guinea-pig, HMT rat, F-344 rat, Sprague-Dawley rat, hamster and mouse.

(ii) Lung clearance of inhaled solid cobalt oxide particles of 0.9 µm diameter, in baboon, dog and HMT rat.

(iii) Interspecies comparison of phagolysosomal pH in alveolar macrophages, using fluorescein-labelled silica particles, in baboon, dog, rabbit, guinea-pig and HMT rat.

(iv) Lung clearance and retention of soluble cobalt after instillation of a Co^{2+} solution, in baboon, dog, guinea-pig, HMT rat and F-344 rat.

In the first two of these studies, translocation of dissolved cobalt

and mechanical clearance of oxide particles were evaluated over at least six months. The particles used varied both in size and in specific surface area. It was concluded that for moderately soluble particles, translocation of the material from lungs to blood was the most prominent clearance mechanism, and was dependent on the species - in man and baboon the rate of translocation was about five times less than in the dog and small animals. Also, particle dissolution was more effective within the alveolar macrophage than in extracellular fluid in the lungs; the rate-determining parameter for translocation was the specific surface area.

The studies on pH in the macrophage phagolysosomes were undertaken to see if this was the determining factor in the difference in translocation rate between the species. However, similar pH values of 4.8 ± 0.2 were found in alveolar macrophages obtained from four species, whose translocation rates in the earlier in vivo experiments had been very different.

Of the dissolved cobalt which is transferred to blood, 5-10% was accumulated in the cartilage of the tracheobronchial airways. This is being compared in the different species to improve the model for translocation of dissolved material, and thereby the general dosimetric model for inhaled radionuclides.

(j) Treatment after Incorporation of Actinides (Reduction of Risk)

The main collaborative effort during the period was directed towards the testing of the catechoyl amide 3,4,3-LICAM(C) for enhancing the elimination of plutonium from the body. The compound had been reported in the literature as being more effective than the current agent of choice, DTPA. The studies involved its administration as either a methylated or pure form to several animal species (mouse, hamster, rat, baboon) after the intake of different chemical forms of plutonium by i.v. injection (citrate) or by inhalation (nitrate and TBP complexes); in the latter case the mass concentrations of plutonium in the lungs were equivalent to those in humans after intakes which ranged between 10 and 10^5 times the ALI. These studies were supported by in vitro experiments designed to investigate the stability of the Pu-LICAM(C) complex at physiological pH and the ability of the chelate to remove plutonium from its carrier protein in blood, transferrin. It was concluded that after the injection of plutonium citrate both forms of LICAM(C) were as effective as DTPA but its potential use in humans was contraindicated by

the excessive amounts deposited in the kidneys; after inhalation of the lower amounts of plutonium as TBP, LICAM(C) was marginally more effective than DTPA, though neither chelating agent under these conditions substantially reduced the body content of plutonium. The enhanced deposition of plutonium in the kidneys was due to the instability of the LICAM complex at about pH 6. The task group recommended that DTPA should remain the agent of choice.

The most promising compounds for the decorporation of plutonium however still appear to be synthetic analogues of siderophores. Collaboration with the University of California and Lawrence Berkeley Laboratory has resulted in advice on the purity of LICAM(C), the supply of two promising compounds (DFO-HOPO and DTPA-DX) which were currently being examined, and detailed information for the EULEP synthesis of a third compound 3,4,3-LIHOPO.

A number of diphosphonate complexes synthesised at the Pierre and Marie Curie University Paris were shown to be inferior to DTPA for enhancing the elimination of plutonium.

During the period 1985-89, improvements were made to the efficacy of DTPA treatment for plutonium by oral administration, infusion and injection. The chelating agent was shown to be most effective for thorium injected intravenously as the citrate and nitrate when administered promptly (1.5 min); the body content was reduced by only about a factor 2 or less when treatment was delayed for more than 30 minutes or when thorium nitrate was inhaled. Tiron, a compound suggested for the treatment of uranium poisoning, was only partially effective for enhancing the elimination of uranium when inhaled in amounts equivalent to the ALI for humans. The development of more effective therapeutic regimes for thorium and uranium will be an area for future research.

(k) Stem Cell Studies after Contamination with Alpha-Emitters

Co-ordinated studies have been designed to investigate the effects of alpha-emitting bone-seeking radionuclides on target cells in mouse bone marrow. In order to interpret metabolic data for such radionuclides, and to provide a rational basis for estimating risk factors for leukaemia and osteosarcoma, it is important to determine the type and location of cells in the bone marrow which would be sensitive to alpha irradiation, as well as their different radiation responses with time.

The radial distribution of stem cells in the mouse femur marrow has

been found to be non-uniform, with the most primitive cells in the axial marrow receiving lower doses from radionuclides in the bone than more mature cells in the marginal marrow. The differential responses of these cells after irradiation with incorporated ^{239}Pu and ^{224}Ra have been compared. Recovery of stem cells took place within three months of Pu administration, but there was a long-term loss in stem cell quality. Recovery was better after Ra treatment, but took place more slowly. The overall pattern of cell migration and radiation response is complex.

Other studies have investigated haemopoietic stem cells in fetal liver and in neonatal spleen and bone marrow following ^{239}Pu administration to the mother at different times of gestation.

It is recognised that the effects of alpha-emitters on the micro-environment of stem cells are also of great importance. This has been studied using long-term bone marrow cultures, irradiated by incorporation of ^{241}Am. Further work has shown that after leukaemogenic doses of ^{239}Pu and ^{224}Ra, different damage to the stem cells and the micro-environment can be seen. Plutonium administered to pregnant mice indicated different target cell populations in the offspring, depending on the time of administration during gestation. The regulation of haemopoiesis by stromal cells was found to be extremely radiosensitive in the offspring following ^{241}Am injection into pregnant mice.

The development of an in vitro assay for bone formation demonstrated the involvement of stromal marrow cells in osteogenic differentiation. This suggests that stromal cells may also be target cells for osteosarcoma development after alpha-irradiation.

(1) Metabolism, Dosimetry and Effects of Bone-Seeking Radionuclides

A major aim of this group is to co-ordinate long-term studies with alpha-emitting radionuclides, which are to determine the metabolism, spatial distribution and toxicity of these materials in bone of various species; the biological end points are leukaemia and osteosarcoma. Thus one collaborative study has investigated plutonium and americium distribution in baboon bones, another has studied ^{226}Ra distribution in beagle dogs. A large life-time study in the CBA mouse has compared ^{239}Pu, ^{241}Am and ^{233}U metabolism in the skeleton. This study showed very different distributions on bone surfaces and in macrophages in the bone marrow at long times after injection. The mice have been given amounts of the three radionuclides which will give the same average dose to the

skeleton. Differences seen in the late effects therefore will be due to differences in the micro-distribution of the alpha-emitters.

One study has highlighted the effect of dose protraction on the incidence of malignant lymphoma: an incidence of 13.5% was observed when ^{224}Ra was injected into the NMRI mouse twice weekly for 36 weeks, giving a final dose of 0.15 Gy. Lymphoma was not seen if the same dose was given from a single injection. More recent studies have shown that whether protraction affects the incidence of osteosarcoma depends on the total amount of activity injected.

In a large-scale study on the effects of low doses of ^{224}Ra in the CBA mouse, it has now been shown that single injections at levels below those which give the maximum incidence of osteosarcoma will induce myeloid leukaemia. In this range of injected activity the incidence of leukaemia is significantly greater than that of osteosarcoma.

Relative risk ratios are also being measured for osteosarcoma in rats, following the administration of ^{238}Pu, ^{237}Np and ^{226}Ra. Preliminary results suggest a risk factor of 12% per Gy for ^{237}Np average alpha dose to bone.

Finally, consideration has been given to effects on man, firstly in a follow-up of patients given ^{224}Ra in the treatment of ankylosing spondylitis. Secondly, recent calculations have shown that the dose to red bone marrow from naturally occurring alpha-emitters may be greater than was thought previously because of the possible concentration of radon in the fat of marrow tissue. The measurement of natural alpha-radioactivity in children's teeth is being used as a method for monitoring environmental exposure in the general population.

(m) Fetal Dosimetry and Effects of Incorporated Radionuclides

This task group was established in 1988 because of an increasing concern regarding the risk to the developing embryo and fetus following the release of radionuclides into the environment. There are no generally accepted models for calculating tissue doses following intakes by the mother, either during or prior to pregnancy. Furthermore, there is only limited information on the radiosensitivity of tissues in the fetus and newborn. Studies are now being co-ordinated in three areas:

(i) The distribution and retention of radionuclides in the fetus and newborn animal compared with that in the mother. A number of laboratories have obtained information on the extent to which

radionuclides are accumulated by the embryo and fetus following acute or chronic intake by the mother at various times before or during gestation. These studies have given information on relative concentrations of radionuclides in maternal and fetal tissues, and the extent to which radionuclides deposit heterogeneously in the embryo and fetus, as well as on the transfer of radionuclides to newborn animals during lactation. Studies have been carried out on a wide range of radionuclides including plutonium, americium, alkaline earths, ^{59}Fe, ^{60}Co, ^{210}Po, ^{125}I and ^{131}I.

(ii) The effects of incorporated radionuclides. A series of studies has been undertaken to assess the consequences of deposition of radionuclides in the developing embryo and fetus, to provide information on the sensitivity of tissues for early as well as late effects, and the extent to which tissue sensitivity varies throughout the period of gestation. In this area the task group has worked closely with the task group on stem cells.

(iii) The development of dosimetric models from animal and human data. The problems in the development of suitable dosimetric models for the fetus include the rapidity of fetal growth, the varying progress of organ development in different species, and the presence of different types of placenta in different species: all of these provide a selective but potentially different barrier between maternal and fetal blood. Effort has been put into developing a dosimetric model for the actinides incorporating information from the different laboratories. This has to take account of the migration of stem cells during fetal life, e.g in the case of haemopoietic tissue. Effort is also going into developing dosimetric models for polonium and radon.

(n) Retention and Absorption of Ingested Radionuclides and Irradiation of the Gastro-intestinal Tract

Since it was formed in 1987, the task group has co-ordinated studies on the gastro-intestinal tract of the neonate, which was considered to be at risk from ingested radioactivity.

Studies of the intestinal retention of plutonium in neonates have demonstrated clear species differences and lead to the conclusion that retention in human neonates is likely to be low. Retention was much greater in neonatal rats than in guinea-pigs: for example, 5 days after administration of ^{238}Pu citrate to 6-day old animals, the small intestine retained about 20% of the administered activity in rats compared with

about 0.02% in guinea-pigs. The cellular distribution of the retained activity is also different, with activity concentrated in the epithelial cells in rats but largely confined to the central region of the villi in guinea-pigs. In the guinea-pigs given ^{239}Pu nitrate at 2 days of age and killed 10 days later, 96% of the dose was delivered to the top 2/3rds of the villi with only about 4% in the sensitive lower region near the crypts. In both species therefore, and particular in guinea-pigs, the retained activity was very largely at some distance from the crypt cells.

The possibility of uptake of ingested particulates into the lymphoid tissue (Peyer's patches) has been studied in suckling guinea-pigs, using particles of plutonium dioxide less than 5 µm diameter. Autoradiographic examination showed very low levels of retention on the villus surface, and no significant retention in the lymphoid tissue.

Limited studies with baboons showed a level and pattern of retention similar to that in the guinea-pig. This suggests that the guinea-pig can be taken as an appropriate model for retention in man. Previous work with neonatal baboons given plutonium nitrate had also shown no evidence of retention in lymphoid tissue.

Work has also been undertaken on the absorption of ingested radionuclides in a human volunteer study to measure the absorption of neptunium and curium, as well as polonium in different chemical forms. Finally, speciation studies are being undertaken to identify the chemical forms of radionuclides in food materials in the gastric and intestinal fluids. The objective of these studies is to determine the availability of radionuclides in the food chain for intestinal absorption.

IV. Other research group(s) collaborating actively on this project [name(s) and address(es)]:

EULEP co-ordinates research on late effects of radiation in 22 Voting Member institutes; it also has over 30 individual corresponding members in other universities and establishments. Through these it is linked to a wide range of academic and research foundations throughout the EC. EULEP has also developed links with three other bodies:

1. European Radiation Dosimetry Group (EURADOS)
2. United States Department of Energy (DOE)
3. European Bone Marrow Transplantation Group (EBMT)

V. Publications:

(This list is limited to items published by or on behalf of EULEP itself.)

EULEP Newsletter: during the period 1985-89, 14 issues have been published (nos. 39-54).

EULEP Pathology Atlas - the following new chapters have been made available:

- Neoplastic and non-neoplastic lesions of the male and female genital tract of the mouse and the rat, 1987.
- Preneoplastic and neoplastic lesions of the urinary bladder of the rat, 1988.
- Neoplastic lesions of the mouse lymphoid system, 1989.
- Preneoplastic and neoplastic lesions of the kidney of the rat, 1989.

EULEP Protocol for X-ray Dosimetry, eds. J Zoetelief, J J Broerse & R W Davies, Commission of the European Communities, EUR 9507 EN, 1985.

EULEP Symposium on Metals in Bone, ed. N D Priest, MTP Press Ltd, Lancaster, UK, 1985.

A H L Aalbers and F J M Bader, The fifth EULEP X-ray dosimetry intercomparison, RIVM report 248005001, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands, 1986.

Symposium on Radiation Carcinogenesis: Molecular and Biological Aspects (co-sponsored by EULEP), eds. J Boniver & M Janowski, Leukaemia Research 10 (7), 1986.

EULEP Symposium on Effects after Combined Exposure to Ionizing Radiation and Chemical Substances, organised by C Streffer, International Journal of Radiation Biology 51 (6), 1987.

DOSELIB: A computer program for the calculation of radiation dose to the population following inhalation or ingestion of radionuclides, D M Taylor and W-G Thies, 1988; floppy disks available on request from IGT, Kernforschungszentrum Karlsruhe.

EULEP Symposium on Skin - its Relevance in Radiological Protection and Radiation Accidents, organised by J W Hopewell in 1989, International Journal of Radiation Biology, in press.

Europäische Gemeinschaften — Kommission
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