ISSN 1018-5593

Commission of the European Communities

radiation protection

Treatment and biological dosimetry of exposed persons

Post-Chernobyl action

Report

EUR 12558 EN

Commission of the European Communities

radiation protection

Treatment and biological dosimetry of exposed persons

Post-Chernobyl action

Edited by:

K. Chadwick, G. Gerber

Commission of the European Communities
200 rue de la Loi
B-1049 Brussels

Final report

Directorate-General
Science, Research and Development

1991

EUR 12558 EN

Published by the
COMMISSION OF THE EUROPEAN COMMUNITIES
Directorate-General
Telecommunications, Information Industries and Innovation
L-2920 Luxembourg

Cataloguing data can be found at the end of this publication

Luxembourg: Office for Official Publications of the European Communities, 1991

ISBN 92-826-3064-1 Catalogue number: CD-NA-12558-EN-C

Printed in Belgium

CONTENTS

Preface

The Chernobyl accident, which occurred on 26 April 1986, presented major challenges to the European Community with respect to the practical and regulatory aspects of radiation protection, public information, trade, particularly in food, and international politics. The Chernobyl accident was also a major challenge to the international scientific community which had to evaluate rapidly the radiological consequences of the accident and advise on the introduction of any countermeasures. Prior to the accident at Chernobyl, countermeasures to reduce the consequences of radioactive contamination had been conceived largely in the context of relatively small accidental releases and for application over relatively small areas. Less consideration had been given to the practical implications of applying such measures in case of a large source term and a spread over a very large area.

The Radiation Protection Research and Training Programme was influential in a number of important initiatives taken within the Community immediately after the accident. Information was collected by Community scientists and, from it, an assessment made within days of the possible consequences. This showed that the health impact on the population of the European Community was not expected to be significant. About four weeks after the accident, the Programme, together with the US Department of Energy, organised a meeting in Brussels during which the data on dispersion of radioactive material were discussed and evaluated. Several other meetings followed soon after on the transfer of radionuclides in the food chain and possible health effects. These meetings were carried out in close co-operation with the DG XI (Directorate General, Environment, Consumer Protection and Nuclear Safety) within the CEC, and, externally, with international organisations such as the International Atomic Energy Agency (IAEA) and the World Health Organisation (WHO). In addition, the Commission convoked a Committee of high-level independent scientists to assess the scientific evidence from current research in view of recent nuclear incidences, to consider the possible implications for the Basic Standards and emergency reference levels and to advise the Commission on future action in radiological protection including research. (EUR 11449 EN).

Soon after the accident, additional research requirements were identified by the Programme; these were mainly better methods to assess accident consequences and

the further improvement of off-site accident management. Several existing contracts were reoriented and new contracts were placed; however, the financial means then available within the Programme were insufficient to fund the additional research identified as necessary. A proposal for a revision of the Programme was, therefore, elaborated in 1986. It comprised 10 specific "post-Chernobyl" research actions. This revision, with an additional budget of 10 MEcu for a period of two years, was adopted by the Council of Ministers on 21 December 1987. With the help of the Management and Coordination Advisory Committee (CGC) "Radiation Protection" a number of institutes was identified to carry out the research in a co-operative manner, and the research began in the spring of 1988.

These post-Chernobyl activities have now been completed. Detailed reports on each of these studies and an additional volume containing the executive summaries of all reports are now available.

- Evaluation of data on the transfer of radionuclides in the food chain,
- Improvement of reliable long-distance atmospheric transport models,
- Radiological aspects of nuclear accident scenarios,
  A. Real-time emergency response systems,
  B. The RADE-AID system,
- Monitoring and surveillance in accident situations,
- Underlying data for derived emergency reference levels,
- Improvement of practical countermeasures against nuclear contamination in the agricultural environment,
- Improvement of practical countermeasures against nuclear contamination in the urban environment,
- Improvement of practical countermeasures: preventive medication,
- Treatment and biological dosimetry of exposed persons,
- Feasibility of studies on health effects due to the reactor accident at Chernobyl.

The research undertaken within the "post-Chernobyl" actions has added considerably to the understanding of the basic underlying mechanisms of the transfer of radionuclides in the environment, of the treatment of accident victims and of how the environmental consequences of accidents may be mitigated. In addition, progress has been made in the setting up environmental surveillance programmes development of predictive and decision-aiding techniques, the implementation of

which will lead to significant improvements in off-site accident management. Several new ideas and lines of theoretical and practical research have originated from the post-Chernobyl research and these have already been integrated into the ongoing Community Radiation Protection Research Programme. A further important feature which should not be overlooked, is the close and effective collaboration of many institutes in the research; this has markedly strengthened the ties between Community institutes and scientists. The outcome of all of this work is that the Community and all other countries are now better prepared and co-ordinated should a significant release of radioactivity ever occur again

Further research is continuing within the current Radiation Protection Research and Training Programme 1990-1991 on a number of the "post-Chernobyl" topics; these also form part of the proposal of the specific Programme on "Nuclear Fission Safety" 1992-1993, e.g. real-time emergency management systems, development of countermeasures in the agricultural environment, treatment of radiation accident victims, etc. Moreover, the Community Programme is currently making a significant contribution to an international evaluation, being undertaken by IAEA at the request of the Soviet Government, on the consequences in the USSR of the Chernobyl accident and of the measures being taken to ensure safe living conditions for the affected populations.

S. Finzi
Director DG XII.D
Nuclear Safety Research

G.B. Gerber
Head of Unit DG XII.D.3
Radiation Protection Research

E. Bennett
Director DG XI.A
Nuclear Safety, Industry and Environment, Civil Protection

Participating Institutions

A) Biological Dosimetry

Institut de Protection et Sûreté Nucléaire
B.P. 6, 92265 Fontenay-aux-Roses Cedex, France

B. Dutrillaux,

Centre d'Etude de l'Energie Nucléaire
rue Charles Lemaire 1, 1160 Bruxelles, Belgique
and Université Catholique de Louvain
Place de l'Université 1, 1348 Louvain-la-Neuve, Belgium

A. Léonard,

National Radiological Protection Board (NRPB)
Oxon OX11 ORQ Chilton Didcot, United Kingdom

D.C. Lloyd, A.A. Edwards,

Universiteit Leiden, Lab. Straalengenetica en Chemische Mutagenese
Wassenaarseweg 72, 2333 Leiden, the Netherlands

A.T. Natarajan, F. Darroudi.

B) Treatment:

Instituut voor Toegepaste Radiobiologie en Immunologie, Hoofdgroep Gezondheidsonderzoek-TNO
Lange Kleiweg 151, 2280 HV Rijswijk, the Netherlands

D.W. van Bekkum, G. Wagemaker,

Universität Ulm, Institut für Arbeits- und Sozialmedizin
Oberer Eselsberg M24, 7900 Ulm, Federal Republic of Germany

T.M. Fliedner,

Institut de Protection et de Sûreté Nucléaire, Dépt. de Protection Sanitaire Service d'Hygiène Radiologique
B.P. 6, 92265 Fontenay-aux-Roses Cedex, France

J.C. Nenot,

Diagnosis and care of radiation accident victims.

Executive summary

1. Introduction

The accident at the Nuclear Power Station at Chernobyl was of great concern to all countries of the E.C. because the radioactive emission was carried over long distances and resulted in variable fall out of radioactive isotopes in all areas of the E.C. Obviously, radiation levels were the highest inside the plant and in its immediate vicinity. At least 200 people involved in the accident itself at the installation or in efforts to control the catastrophe by bringing the reactor under control were exposed to radiation levels and/or other injuries of such extent that immediate medical care was required. The information concerning the acute victims, that was released by the Russian authorities and the subsequent results of the medical interventions proved to be of great interest to radiation experts of the E.C. Although the radiation protection program of the E.C. had for many years included applied research on the effects of high dose radiation exposure and on the treatment of its consequences, the analysis of the acute Chernobyl cases revealed several new issues, not foreseen from the experience of past reactor accidents, that were all of a much more limited extend.

These problems were analysed by experts from the Advisory Committy as the data became available and the most important ones were selected for study in the context of a crash research effort, that is designated as the Post- Chernobyl program.

The result of the projects concerned with diagnosis, prognosis and treatment of acute radiation injuries are reported in this chapter.

2. The experience of Chernobyl

During the first hours and or days of the accident, several hundreds of persons were exposed to extensive radiation from the burning reactor and its emissions. A certain proportion of the victims had also suffered thermal burns and inhalation of radioactive isotipes of unknown concentration and amount had occured as well. In the majority of the cases the body dose received by individuals could not be reliably derived form personal dosimeters carried by the victims. On the bases of calculated exposures, using time, duration of presence and location, about 200 persons were selected for immediate hospitalisation on the likelyhood of need for treatment. They were transported to specialised hospitals in Moskou and Kiew for further diagnostic and prognostic evaluation. On the bases of peripheral blood counts, bone marrow puncture and chromosomal analysis, 19 patients were treated with bone marrow or fatal liver cell transplants. The clinical course and the results have been reported in a general way only and the medical community is still awaiting more detailed information. Anyhow the effects of the transplantations have been disappointing in that a benificial influence was not observed.

In part this seems to be due to the mixed type injuries suffered by the victims (in particular skin burns and intestinal damage in combination with hemopoietic injury). Other factors were the lack of HLA identical sibling donors of bone marrow and the fact that there had been no experience with bone marrow transplantation in Russia.

This was also the first occasion that a large number of heavily irradiated people had to be treated at the same time in one hospital. Owing to the numbers of victims involved and the nature of the radiation exposure, these observations are obviously of great interest to the Commission on Radiation Protection of E.C.

Within the first few days after the accident, the Commission approached the various bone marrow transplantation teams in the E.C. and made an inventory of the number of beds that could be made available for Russian patients possibly requiring treatment with bone marrow grafts. The Russian authorities were then offered to transport any number of transplant candidates to these highly specialised teams in Europe, but this proposal was not accepted.

During the months after the accident, experts of the E.C. met on a number of occasions to evaluate the data coming from Russia and to organise an emergency program for research into the new and unforeseen questions arising from the Chernobyl experience.

3. E.C. crash program on prognostics and treatment of heavily exposed radiation victims.

The main problem was the great uncertanty in establishing the risk of the individual patient to develop a fatal radiation syndrome. The currently available biological indicators were lowered peripheral white

blood cell counts, increased chromosomal abnormalities and the severity and rate of development of clinical sickness shortly after exposure. None of their parameters provides a very accurate risk estimate. Therefore, it was decided to reanalyse the predictive value of the changes in blood cell counts and to perform in vitro studies on dose effect relations of chromosome aberrations with emphasis on the influence of partial body irradiation. In addition, a new approach to biological prognostic dosimetry was initiated by studying the possible application of the new Hemopoietic Growth Factors (HGF) for determining the amount of surviving stem cells of the blood forming system. These stem cells are the only cells that are important for the recovery of the blood formation. They may be activated and stimulated by the hormonal action of the HGF to produce progency, which should reflect in the peripheral blood cell counts. If insufficient stem cells are surviving, administration of HGF would not result in a response and this would then indicate a poor prognosis.

For patients with such damage to the blood forming system as was hitherto considered irreversible, the recommended treatment was bone marrow transplantation. However, bone marrow transplantation is thus far effective only when the recipient has been subjected to homogeneous irradiation of the whole body and when bone marrow from a matched donor is available. It appears that these conditions were not fulfilled for the majority of the reactor accident victims.

Inhomogeneous exposure may leave a small proportion of the white blood cells responsible for the bodies defence, intact, which is sufficient for a bone marrow graft from another person to be rejected.

The new HGF seemed to provide possibilities for stimulating residual autochtonous stem cells following inhomogeneous irradiation or perhaps

even following an otherwise lethal dose of homogeneous exposure, and thereby induce cures by endogeneous regeneration of the blood forming system. Such investigations can obviously not be carried out with patients, but it is feasible to obtain certain essential pieces of information like the of doses HGF required for a substantial response, or the occurence of toxic side effects, from studying patients with hematological diseases who are being treated with HGF. Other and more essential data can only be acquired from experiments with subhuman primates, which greatly resemble humans in their responses to irradiation.

4. Result from the program

Prognostic value of blood cell counts

The Ulm Research group has established a Radiation Accident Data Bank in which all available data on changes in blood cells and blood cel forming tissues are being collected, in conjunction with clinical symptoms, the course of radiation sickness if developing and its eventual outcome. A total of 22 radiation accidents, resulting in the exposure of the whole body or a large part of the body and involving over 600 individuals is being analysed. At the time of this report more than 300 cases have been processed and results of calculations based on the counts of peripheral blood cells over the observation period have been related to the physically estimated dose of acute total body irradiation.

A computer based simulation model of the formation of one class of white blood cells (the granulocytes) has been designed which allows the

calculation of the number of intact blood forming stem cells that survive the irradiation. The results so far suggest that a calculated surviving fraction of stem cells of 8×10^{-6} of the normal number is the minimum that will allow spontanous regeneration of the blood formation.

The Rijswijk group of researchers employed a somewhat different approach to arrive at minimal surviving stem cells numbers compatable with spontanous recovery. They compared in rhesus monkeys the time period required for spontanous recovery of peripheral blood cells after total body irradiation with a range of doses with the time of recovery seen in lethaly irradiated monkeys grafted with graded numbers of autologous bone marrow cells. Spontanous recovery occured in monkeys with a calculated surviving fraction of 10^{-5} of normal, which is very simular to the value for humans of 8×10^{-6} referred to above. However, the dose of total body irradiation which corresponds to that surviving fraction was 11.5 Gy gamma radiation in the monkeys, while in the patients studied by the Ulm group the corresponding dose was 5 Gy at most. However, only 2 patients were available in the dose range between 10 and 12 Gy and the data between 10 and 11,5 Gy total body irradiation were derived from 4 monkeys only. The monkey data revealed that the surviving fraction of stem cells at 5 Gy total body irradiation was 10^{-3}.

Clearly, there are important as yet unresolved differences between the results obtained from the experiments with monkeys and those derived from human victims of radiation accidents. It can not be excluded that the human stem cells are more radiosensitive than those of the monkey. The results with monkeys have clearly shown that regeneration of leukocyte numbers relate less dependably to the radiation dose than

regeneration of reticulocytes, the former being disturbed by the occurrence of infections. It is advisable to take this into account in the interpretation of the granulocyte counts in humans. The most important problem however is so far the absence of human data for doses between 5 and 10 Gy and it is urgent to collect those, if available. The major advantage of the approaches reported here is that the prognostic parameters employed are not dependent on homogeneity of the exposure, since they relate to the number of surviving stem cells irrespective of the location of those cells.

5. Prognostic value of administration of Hemopoietic Growth Factors (HGF).

Recombinant HGF have been evaluated as a means to determine the number of surviving stem cells in irradiated subjects. The main data were obtained with GM-CSF and interleukin 3. Since a species barrier was discovered for r. hum. IL-3, it was necessary to produce r. Rhesus IL-3 for the studies in irradiated monkeys. This was accomplished by the Rijswijk group under this program.

The French team at Fontenay aux Roses has made extensive studies in vitro on GM-CSF added to long term human bone marrow cultures. They observed no adverse effects on either the stromal cells or on the proliferation capacity of hemopoietic precursor cells. GM-CSF has a broad range of actions on hemopoietic progenitor cells, including pluripotent stem cells. It stimulates the proliferation of cells in many of the differentiated lineages. Therefore it was investigated whether early post-irradiation treatment with GM-CSF might cause a detectable response of the peripheral blood cell counts and whether the

capacity for endogeneous hemopoietic recovery could be reliably determined by such a response.

It was found that GM-CSF can indeed serve that purpose in the dose range between 4 Gy and 8 Gy (6 MEV X-rays). However, the variations are considerable and at least 8 days of observation are required for a reaction. Responses were not seen after doses greater than 8 Gy (more than 4 log cell kill), although the ressidual stem cell numbers are sufficient for endogeneous regeneration. Preliminary data with IL-3 treatment of irradiated monkeys are simular to those seen with GM-CSF. Therefore it has to be concluded that treatment with these factors cannot result in a clear cut distinction between those who will, and those who cannot regenerate spontanously.

6. Biological dosimetry by quantitative analysis of chromosomal damage.

a) As chromosomal analysis is particularly labour intensive and time consuming there are considerable logistical problems in dealing with large numbers of patients. The new micronucleus assay that utilises cytochalasin B to block cytokinesis offers an alternative to scoring for dicentrics. Because the images are far simpler, the analysis could be completed significantly faster and by less skilled technicians. This technique was not available to the Soviet laboratories that responded to the Chernobyl accident.

There is usually an urgent need to provide an estimate of dose as quickly as possible. The present techniques are not ideal in this respect because for dicentric analysis the microscopy cannot begin until 2 days after receipt of the blood sample. This is because the

lymphocytes need to be stimulated with a mitogen and cultered for 48 hours so that they can be analysed at first metaphase. For the micronucleus assay the culture time is even longer, 72 hours; although as indicated above once the preparations are made the analysis time is much shorter. In practice therefore a dose estimate is not available until three or four days after receipt of a blood specimen.

The relatively new method of Prematurely Condensed Chromosomes (PCC) however opens up the possibility of scoring aberrations within a few hours of blood sampling. The technique is maintained at present as a routine test only in Leiden. It has the potential for overcoming the complications of interphase death and mitotic delay which apply to conventional metaphase analysis with no loss of sensitivity or accuracy. The technique, being new, does require further background research.

b) The Chernobyl experience was notable for highlighting two important features of biological dosimetry that are particularly relevant for high life-threatening exposures. Firstly, for highly overexposed subjects, lymphocyte cultures yield a low mitotic index and there are two reasons for this; (a) the rapid decline in the numbers of lymphocytes in the peripheral circulation and (b) highly irradiated cells respond in culture less readily to mitogens and proceed more slowly around the cell cycle to metaphase. Thus in order to increase the number of cells available for analysis there is a need to improve the mitotic index in such circumstances and to quantify the mitotic delay. Secondly, accidental irradiation is usually inhomogeneous and this can often be detected cytogenetically because the distribution of

aberrations among the scored cells is overdispersed with respect to the Poisson distribution that characterises an uniform whole body exposure.

c) Acute partial body irradiations to 5 or 8 Gy x-rays were simulated at Chilton by in vitro irradiation of blood which was then mixed with unirradiated blood from the same donor. The irradiated fractions of the mixtures ranged from 0.5 to 0.1. The mixtures were coded for blind analysis and shared between Chilton and Leiden and cultured for chromosomal aberrations and micronuclei in cytokinesis blocked cells. In Leiden only, prematurely condensed chromosomal fragments were analysed in mixtures of irradiated and non-irradiated cells.

d) The analysis applied to the dicentric distributions scored at both Leiden and Chilton showed that for most mixtures both the estimates of the irradiated fraction and their doses agreed well with the true values. It was noted that even for 95% of cells irradiated at 5 Gy there were still about 35% of cells seen that did not contain any aberrations and so were apparently undamaged. At 8.0 Gy and 99% irradiated about 20% of cells seen were normal. By contrast for 100% irradiated at both doses no cells were seen to be free from aberrations. This information in itself is of immediate value as an indication that some proportion of the cells has been spared. The implication from this is that there is likely to be natural recovery of bone marrow. For the analysis for a highly irradiated subject this would be apparent after relatively few cells had been scored.

e) Culture techniques were extensively compared between the participating teams in Paris, Brussels, Leiden and Chilton and this has

resulted in notable improvements and optimal standardisation. Two relativily new methods were also investigated in depth: the micronuclear technique and the assay based on prematurely condensed chromosomes. The latter technique turned out to yield comparable values to those of the estimation of dicentric chromosomes and to possess several important advantages: results can be scored within a few hours of blood sampling and there is no interference from interphase death and mitotic delay.

7. Therapeutic implications.

Our present inability to arrive at a dependable estimate of the number of residual stem cells at low stem cells numbers,i.e. at high doses of irradiation, implies that a reliable distinction can not yet be made between subjects with a high probability of spontanous recovery and those with a low probability. The experiments with rhesus monkeys indicate that spontanous hemopoietic regeneration can always be expected provided that intestinal radiation damage is sublethal. In the homogeneously irradiated monkeys there was no significant dose interval between spontanous recovery and death from intestinal damage. If these observations apply to humans, it should be safe not to attempt treatment with bone marrow transplants, in view of the fact that in accident cases more marrow is spared because of the inhomogeneous distribution of the dose over the body. Treatment with the hemopoietic growth factors GM-CSF, possibly in combination with IL-3 is recommended to be started soon after the exposure in all patients who are expected to develop pancytopenia. Such treatment will shorten the pancytopenic period and reduce the associated risks in patients who will eventually

recover spontanously and it is not expected to do any harm to patients who cannot regenerate from their residual stem cells.

Indications for a substantial degree of inhomogeneous exposure can be obtained from dicentric chromosome counts and at an early time after the exposure from the PCC technique.

Such indications when obtained with high dose estimates, favour the probability of endogeneous recovery and should discourage the use of bone marrow transplantation. The absence of signs of inhomogeneous exposure in conjunction with high dose should favour attempts at rescue with bone marrow grafts. In such cases, perhaps very rare, rejection of the allogeneic graft is much less likely than in case of inhomogeneous exposure, when surviving lymphatic cells can react against the graft. In case strong indications are present for bone marrow transplantation, the chances of finding an HLA identical sibling donor will be small. Other donors are not recommanded because of the high risks of rejection or fatal graft versus host disease.

Most of these dilemmas would be avoided in case the patients own unirradiated bone marrow were available for reinfusion.

Collection and storage of bone marrow from nuclear plant personnel prior to an accident is not feasible. On the other hand, collection of stem cells from the peripheral blood of personnel that is sent on rescue missions is worth considering.

8. Recommendations

The duration of the special post- Chernobyl research projects has obviously been insufficient to solve all problems that were selected for study. However, the results thus far obtained have permitted a

number of conclusions to be drawn regarding further research and organisational measures of high priority. These concern problems related to diagnosis and prognosis, treatment and logistics of operations following the identification of accident victims.

a. Diagnostic procedures with prognostic relevance

Further analysis of the prognostic significance of blood cell counts is reguired in particular for total body doses of between 5 Gy and 12 Gy. For these purposes the data bank operated at Ulm and the mathematical models of myelopoiesis should be further expanded.

The results so far obtained with GM-CSF administration as a means of evaluating the size of the surviving hemopoietic stem cell compartment should be continued with IL-3 and combinations of IL-3 and GM-CSF. Separate studies should be carried out in rhesus monkeys subjected to inhomogeneous total body irradiation, with and without factor administration.

The techniques to determine chromosome damage by counting of dicentrics and prematurely condensed chromosome estimates in particular have to be established in a larger number of European Laboratories than were involved in the present study. Training courses have to be organised to disseminate the PCC technique. Seperate investigations are needed for the evaluation of these methods in determining the degree of inhomogeneity of the exposure, by setting up in vivo experiments preferably with subhuman primates.

Finally, increased efforts are recommended to achieve a better understanding of the interaction between extensive thermal and or

radiation damage to the skin and the damage to hemopoietic tissues so that prognostic parameters in cases of combined injury may be obtained. For all the methods referred to above it is necessary to increase our knowledge about their usefullness in cases of protracted exposure to radiation.

b. Improvement of therapeutic means

Further research both in preclinical animal models and in patients is needed to improve the therapeutic potential of hemopoietic growth factors. Combinations of factors and variations of the doses have yet been insufficiently explored. Also their therapeutic value in cases of inhomogeneous exposure has to be established in vivo. The use of HGF in combination with allogeneic bone marrow transplantation also deserves much attention. It is possible that HGF would promote the proliferation of the grafted cells and thereby overcome the problems of graft rejection imposed by inhomogenous exposure. In this respect it is important to investigate in monkeys whether the residual immune capacity following inhomogeneous exposure may be neutralised by pretreatment of the patient with appropriate anti lymphocyte antibodies, as they have been shown in rodents to allow a reduction of approximately 2 Gy of the total body dose required for bone marrow takes. Similar studies are recommended with some of the new immunosuppressive drugs. The importance of continued research on the use of autologous stem cells derived from the peripheral blood can hardly be overestimated. If safe and reliable technology could be developed for harvesting an adquate number of stem cells from the

peripheral blood of healthy people, this would be a method of choice to protect individuals at risk in the case of a nuclear accident.

c. Organisation and logistics

Although there is presently an increasing number of well equiped hospitals where patients are treated with high dose total body irradiation and bone marrow transplantation, on a regular basis, the medical staffs in this places are not usualy trained in the handling of victims of radiation accidents. It is recommended that the Commission takes the initiative for organising courses in Europe to bring these medical staffs up to date, in particular with regard to the handling of contaminated patients and to the medical problems arising in patients with combined injuries.

It is recommended that these hospitals establish formal connections with laboratories capable of peforming analysis of chromosomal damage, so that immediate action can be taken in case of exposed individuals.

To support such a network of "stand by" medical and laboratory facilities to be used in case of accidents, an expert system should be established that is available to all. A special effort is required to establish such an international (European) expert system and to guanrantee its maintainance by adding pertinent new information as it becomes available. An exellent basis for this expert system is obviously provided by the data bank of radiation accidents which has been established in Ulm.

Finally it is recommanded that basic research on the effects of ionising radiation on the hemopoietic system and factors that modify this damage continues to be stimulated by the Commission.

COMMISSARIAT A L'ENERGIE ATOMIQUE

INSTITUT DE PROTECTION ET SURETE NUCLEAIRE

CENTRE D'ETUDE DE L'ENERGIE NUCLEAIRE

UNIVERSITE CATHOLIQUE DE LOUVAIN

CONTRACT BI6 - 0283 - F
CONTRACT BI6 - 0284 - B
CONTRACT BI6 - 0320 - B

TECHNICAL IMPROVEMENTS OF THE CYTOGENETICAL METHODS USED FOR BIOLOGICAL DOSIMETRY IN CASES OF A CHERNOBYL-LIKE ACCIDENT

B. DUTRILLAUX, A. LEONARD

I. Objectives of the project :

Biological dosimetry based on the observation of chromosome aberrations in peripheral blood lymphocytes is performed by relatively few laboratories. Since accidental exposure to high doses of ionizing radiation results in a drastic decrease of lymphocytes in the peripheral blood, the cultures are always made in very rich medium which provides higher number of dividing Ml 44-48 h after initiation of the cultures. The chromosomes, however, are more contracted but anomalies such as dicentrics are easily detected. The low number of laboratories doing biological dosimetry in routine contrasts with the numerous units using cytogenetic methods for medical purposes. The culture medium they employ is relatively poor and the number of metaphases obtained after 48 h of cultivation is low. The method, however permits the obtention of elongated prometaphasic chromosomes particularly appropriate to high resolution banding.

The first part of the programme had the aim to improve the methods of cell synchronisation which could highly increase the number of dividing and analysable cells and which could also be easily applicable by medical cytogenetic laboratories. In the eventuality of a serious nuclear accident it could be necessary, indeed, to perform biological dosimetry on a large number of presumably exposed individuals within a few days following the accident, a programme which would largely exceed the capacities of the few existing specialized laboratories.

The second part of the proposal was a complement to the study of Dr.D.LLOYD (Chilton) and Dr.NATARAJAN (Leiden) on the problems resulting from inhomogeneous or partial body exposure to ionizing radiation. Using a chromosome marker to distinguish irradiated cells from non-irradiated ones an attempt was made to estimate the influence of mitotic delay resulting from irradiation and of the dilution of the irradiated cells on the dose as estimated by the incidence of chromosome anomalies.

II . Progress achieved

(1) Cellular synchronisation

Methodology

The synchronisation of the lymphocytes was obtained by addition of thymidine to the culture medium, a treatment reported to increase the number of dividing cells (Viegas-Pérignot and Dutrillaux, Ann.Génét., 21, 122-125, 1978).

In Fontenay-aux-Roses, 0.3 to 3 mg/ml thymidine was added 24 to 36 h after the initiation of the cultures in TC199 medium additioned of 20 % human serum, phytohemagglutinin and antibiotics. This culture medium is currently used by laboratories specialized in medical cytogenetics. The block was released 7-8 h before harvesting by the addition of 10 µg/ml 2-deoxycytidine. The duration of the thymidine block varied from 14 to 48 h. In some cultures, 5-bromodeoxyuridine (BrdU) was added to the medium in order to evaluate the number of cell devisions having occured "in vitro". A fluorescence plus Giemsa (FPG) treatment was used to detect BrdU incorporation. Various treatments by colchicine (1 to 5 h) were applied to define the conditions giving the highest mitotic index compatible with a good quality of the metaphases. Doses of γ-radiation delivered by a cobalt-60 source ranged from 1 to 10 Gy.

In Brussels, blood samples from two donors were given 0, 1, 3, or 6 Gy of X-irradiation and were incubated in Ham's medium, alone or supplemented with 300 µg thymidine/ml culture medium. After an overnight exposure to thymidine, the blocking of the cell cycle was reversed by the addition of 0.5 mg deoxycitidine/ml culture medium. In order to study the cell kinetics, some cultures were supplemented with BrdU at a dose of 0.05 mg/culture. The culture time was 48 h for cells incubated without thymidine and 53 h for those cells cultivated with

thymidine. In a second set of experiments performed according to the same protocol, the cultures were additioned with 600 g thymidine/ml culture medium. Additional experiments were performed to study the effect of the duration of exposure to colchicine (3 to 5 h) and of the culture time (56 to 62 h) on the mitotic index, on the number of analysable cells and the cell cycle of human lymphocytes cultivated in Ham's medium containing 300 µg thymidine/culture.

Results

CEA, Fontenay-aux-Roses

Whatever were the conditions, all metaphases were in M1 after synchronization without thymidine, the rate of M2 and M3 ranging from 5 to more than 60 % after culture duration of 48 and 60 h respectively without synchronisation. As the blocking induced by thymidine was clearly efficient a 53 h culture time was selected for the other experiments.

In almost all conditions, mitotic index was increased by thymidine block.

The effect of the dose of thymidine was studied in a series of 4 donors, and the dose of 0.3 mg/ml finally was selected. Increase of the duration of treatment by colchicine did not result in a strong condensation of the chromosomes but increased the mitotic index and a 4 to 5 h treatment by colchicine was used in further experiments.

Two protocoles for releasing thymidine blocking were compared i.e. addition of 2-deoxycitidine and to change the culture medium and to rince the cells. Very similar results were obtained. For this reason the following protocol, cheaper and less time consuming was adopted :

- Time 0 : initiation of the cultures (TC 199 medium + human serum + antibiotics + PHA) ;

- Time 24 to 30 h : addition of thymidine (0.3 mg/ml) ;

- Time 45 h : addition of 2-deoxycitidine (0.01 mg/ml) ;

- Time 49 h : addition of colchicine ;

- Time 53 h : harvest.

Using this protocol, mitotic index ranging from 30 to 60 per 1000 was obtained in non irradiated cultures, all metaphases being in M1. Such mitotic indexes were several folds higher than those usually obtained without thymidine. In addition, the quality of the metaphases was greatly improved.

After exposure to ionizing radiations, all metaphases were in M1 and treatment with thymidine had comparable effects : increase of mitotic index by 4 to 5 fold, improvement of the quality of metaphases.

CEN and UCL, Brussels

. . . . . .

The results of the observations are summarized in Tables I-VIII. They show that :

- a dose of 300 µg thymidine/ml culture medium increases generally the number of analysable metaphases (Table I) but does not modify the cell kinetics (Table II) ;

- a dose of 600 µg thymidine/ml culture medium is much less effective with respect to its effect on the number of analysable metaphases (Table III) and could be toxic as suggested by its effect on cell kinetics (Table IV).
- the increase of the duration of exposure to colchicine from 3 to 5 h does not modify greatly neither the mitotic index nor the number of analysable metaphases (Table V) ; the cell kinetics was not modified all cells being in M1 (Table VI) ;
- increase of culture time from 56 h to 62 h does not influence neither the mitotic index, nor the number of analysable metaphases nor the cell kinetics (Tables VII and VIII).

(2) Influence of the radiation-induced mitotic delay and of the dilution factor on the dose estimate

Biological dosimetry based on the observation of chromosomes in peripheral blood lymphocytes provides only an estimate on of the average damage caused to the entired lymphocytes population. One of the major difficulties results from the inhomogeneity of the dose and of partial body exposure. In addition, selective elimination or lower proliferation rate of irradiated cells can lead to an important underestimate of the dose.

In the present study evaluation of survival and kinetics of irradiated cells was made on mixtures of male irradiated blood lymphocytes and female control ones, the Y chromosome being used as marker. An attempt was made to calculate the dose inhomogeneity on the basis of the dicentric distribution and its deviation from Poisson's law.

Methodology

Fontenay-aux-Roses

The two donors, one male and one female, were selected on the basis of their high number of lymphocytes per ml of blood (4575 and 3744 respectively) and of their similar response to PHA stimulation. In mixed cultures, the percentages of metaphases of each sexe were roughly proportional to the respective quantities of male and female blood which were seeded. The blood from the male donor received 0, 1, 2, 4, 6, or 10 Gy γ-rays from a cobalt 60 source.

Aliquots of irradiated blood were mixed to female blood samples in the proportions 1:0, 1:1, 1:4, 1:9 and 1:19 before to be put in culture in TC 199 medium. Two types of cultures were developed, using or not synchronization technique. In addition, in the non irradiated series, BrdU was added in order to study cell cycles, in the various conditions of dilution. Cultures without synchronization were prolonged for 48 h, and those with synchronization for 53 h, including a 21 h treatment by thymidine, followed by addition of 2-deoxycytidine (last 8 h) and colchicine (last 4 h). Slides were stained by Giemsa, except for those obtained after BrdU treatment which were stained by a modified FPG treatment. Except for a few cases, 200 metaphases were analysed for each experimental condition. Mitotic indexes were calculated by examining 1000 nuclei at least, and 100 metaphases were analysed for the study of cell cycles.

Brussels

The experiments were performed with blood samples from two donors selected also on the basis of a high and comparable number of lymphocytes and similar response to PHA stimulation.

Male human peripheral blood samples were exposed to 0, 1, 2, 4, 6 or 10 Gy of X-irradiation by means of a Philips RT 250 X-ray apparatus operating at 250 kV, 15 mA, 0.5 mm Cu filtration and a dose rate of 1 Gy/min. Before culturing, the irradiated and non-irradiated male blood were mixed in different ratios (1:0, 1:1, 1:4, 1:9 or 1:19) with non-irradiated blood from the female donor. Culture were made with and without synchronisation by thymidine.

Results

Figures 1 to 6 summarize the results obtained in Fontenay-aux-Roses, figures 7 to 12 and tables IX to XXVII, the results obtained in Brussels.

As expected, after culture in TC 199 medium, the mitotic index and the proportion of analysable metaphases were greatly increased by thymidine block. Quite surprisingly was the fact that, mainly in the cultures treated with thymidine block, the proliferation of the female non-irradiated cells was increased by the addition of highly irradiated (6 and 10 Gy) cells. The observations on the mixtures of irradiated male cells and non-irradiated ones also confirmed the numerous reports indicating that after irradiation plasma contains a clastogenic factor induced or activated by the radiation. For instance, in 10 Gy radiation series 12 to 20 % female metaphases contained chromatid lesions in synchronized cultures. These percentages ranged from 5 to 12 in non-synchronized cultures. Similar effects of irradiated blood on non-irradiated female cells were observed after culture in Ham's medium but were less marked.

The mean numbers of dicentric and ring chromosomes and of chromosome breaks per abnormal metaphase were fairly independent from the dilution and, as expected, were directly correlated to the dose administered. For cells cultivated without thymidine in Ham's medium the average numbers of dicentric and centric ring

chromosomes and breaks per abnormal cells were respectively 0.6 and 1.6 after a Gy, 0.9 and 2.04 after 2 Gy, 1.0 and 3.25 after 4 Gy, 2.3 and 5.75 after 6 Gy, 3.3 and 8.4 after 10 Gy (Tables IX to XII). Comparable results were obtained with cells cultured in TC 199 medium. Very informative also were the maximum number of breaks and the maximum number of dicentrics (Tables XVII to XXVI) observed in abnormal metaphases which were directly related to the doses.

Irradiation induced a dose-related mitotic delay which resulted in differences between the numbers of dicentrics expected and observed. This effect was apparently not influenced neither by the nature of the culture medium nor the treatment by thymidine. It was, however, depending on the dilution mainly when the proportion of irradiated cells to non-irradiated ones was low (1:9 and 1:19) : this observation suggests that the probability for an irradiated cell to divide is lower when it is lost among numerous unirradiated ones (Tables XIII and XIV).

Dicentrics produced in male cells non-mixed with female unirradiated ones followed the Poisson distribution, the only exception being the cells given 10 Gy and cultivated in the presence of thymidine (Tables XIV and XV). As expected the deviation of the yield of dicentric and ring chromosomes from the Poisson distribution was significant when irradiated cells were mixed to female unirradiated ones (U test, Papworth, 1970). Tables XVII to XXVII demonstrate that the contaminated Poisson method described by Dolphin (1969) can provide an very good estimation of the local dose administered and an acceptable one of the fraction exposed. Since no overdispersion was observed in cells exposed to 1 Gy estimate of the fraction of cells irradiated was not possible for that dose.

Conclusions

1. Cell synchronisation

Synchronization improves cytogenetic analysis at various degrees, depending on medium used. As far as a generalisation can be done, culture conditions developed by laboratories specialized in dosimetry like those of Brussels have been optimized, by the use of very rich medium. This gives a relatively high mitotic index, but a relatively low quality of chromosomes, as regards to their elongation permitting banding analyses. In these conditions, the slight improvement brought by synchronization (better quality of metaphases, no second generation) may not justify the additional manipulations required.

The situation is quite different for other cytogenetics laboratories, since the quality (i.e. elongation) of the chromosome is the first criteria. In most of these laboratories, the medium used is not very rich, and the cell progression not fast. Synchronization without treatment by colchicine permits the obtention of prometaphasic chromosomes favorable for high resolution chromosome banding and is currently used. The modification proposed here permits to obtain mitotic index at least as high as this obtained by laboratories specialized in biological dosimetry. All metaphases are at first generation in vitro, and the quality of their chromosomes better.

In case of accident most cytogenetic laboratories can very easily adopt the technique proposed here. This is certainly more realistic than to ask for complete change of culture conditions and the method proposed here may permit a high increase of the cytogenetic potential, in case of a severe accident.

In summary :

1) In laboratories specialized in dosimetry based on chromosome observation synchronization increases the quality of the metaphases, but it does not change mitotic index.

2) Since the technique is more time consuming, it probably does not correspond to a real improvement for specialized laboratories.

3) In non specialized laboratories, synchronization technique clearly increases mitotic index to the level of that obtained by specialized laboratories. The quality of chromosome spreads is much improved. This technique can be very easily adapted by any laboratory working on medical diagnosis.

2. Influence of the radiation-induced mitotic delay and of the dilution on the dose estimate

The use of the Y chromosome to distinguish male cells irradiated with increased doses and mixed in different proportions with unirradiated female ones have demonstrated that :

- the mean and maximum numbers of dicentric and ring chromosomes and of break are clarely dependent on the doses but are not influenced by the dilution ;
- the degree of dilution influences the probability of an irradiated cells to be observed in metaphase ;
- the contaminated Poisson method can provide a good estimate of the dose received locally and, to some extent, of the fraction of the body which has been exposed.

Table I.

Mitotic index and analysable metaphases per 1 000 stimulated cells in the presence or absence of a thymidine block (300 µg/ml culture medium).

Dose (Gy)	With thymidine		Without thymidine	
	Mitotic index	Analysable cells	Mitotic index	Analysable cells
0	133	49	147	30
1	133	40	126	21
3	82	21	97	21
6	106	26	57	13

Table II.

Cell kinetics (M1 and M2 per 100 cells)of lymphocytes cultivated in the presence or absence of a thymidine block (300 µg/ml culture medium).

Dose (Gy)	With thymidine		Without thymidine	
	M1	M2	M1	M2
0	78	22	73	27
1	81	19	74	26
3	87	13	85	15
6	93	7	92	8

Table III.

Mitotic index and analysable cells per 1 000 stimulated cells in the presence or absence of a thymidine block (600 µg/ml culture medium).

Dose (Gy)	With thymidine		Without thymidine	
	Mitotic index	Analysable cells	Mitotic index	Analysable cells
0	69	41	63	34
1	42	23	35	19
3	40	20	36	22
6	18	10	13	5

Table IV.

Cell kinetics (M1 and M2 per 100 cells) of lymphocytes cultivated in the presence or absence of a thymidine block (600 µg/ml culture medium).

Dose (Gy)	With thymidine		Without thymidine	
	M1	M2	M1	M2
0	92	8	72	28
1	97	3	82	18
3	98	2	92	8
6	99	1	95	5

Table V.

Influence of the duration of the exposure time to colchicine on lymphocytes cultivated in the presence of a thymidine block (300 µg/ml culture medium).

Cells were harvested 52h45 after initiation of the cultures.

Dose (Gy)	Duration of the colchicine treatment					
	3 h		4 h		5 h	
	Mitotic index	Analysable cells	Mitotic index	Analysable cells	Mitotic index	Analysable cells
0	127	30	124	48	162	45
1	106	30	104	13	152	30
3	105	20	92	16	105	32
6	89	15	103	31	75	13

Table VI.

Influence of the duration of the exposure to colchicine on the cell cycle of lymphocytes cultivated in the presence of a thymidine block (300 µg/ml culture medium). Cells were harvested 52h45 after initiation of the cultures.

Dose (Gy)	Duration of the colchicine treatment					
	3 h		4 h		5 h	
	M1	M2	M1	M2	M1	M2
0	100	-	99	1	100	-
1	99	1	100	-	99	1
3	100	-	100	-	100	-
6	100	-	99	1	100	-

Table VII.

Cell kinetics after different cultivation times (Thymidine 300 µg/ml ; colchicine, 3 h).

Dose (Gy)	Cultivation time					
	56 h		59 h		62 h	
	M1	M2	M1	M2	M1	M2
0	99	1	99	1	99	1
1	99	1	100	-	99	1
3	99	1	100	-	99	1
6	100	-	100	-	100	-

Table VIII.

Mitotic index and analysable cells in 1 000 stimulated cells after different culture time (300 µg/ml thymidine ; colchicine, 3 h).

Dose (Gy)	Culture duration					
	56 h		59 h		62 h	
	Mitotic index	Analysable cells	Mitotic index	Analysable cells	Mitotic index	Analysable cells
0	58	15	38	10	41	11
1	72	34	37	5	32	10
3	43	4	39	8	42	15
6	33	8	35	11	24	7

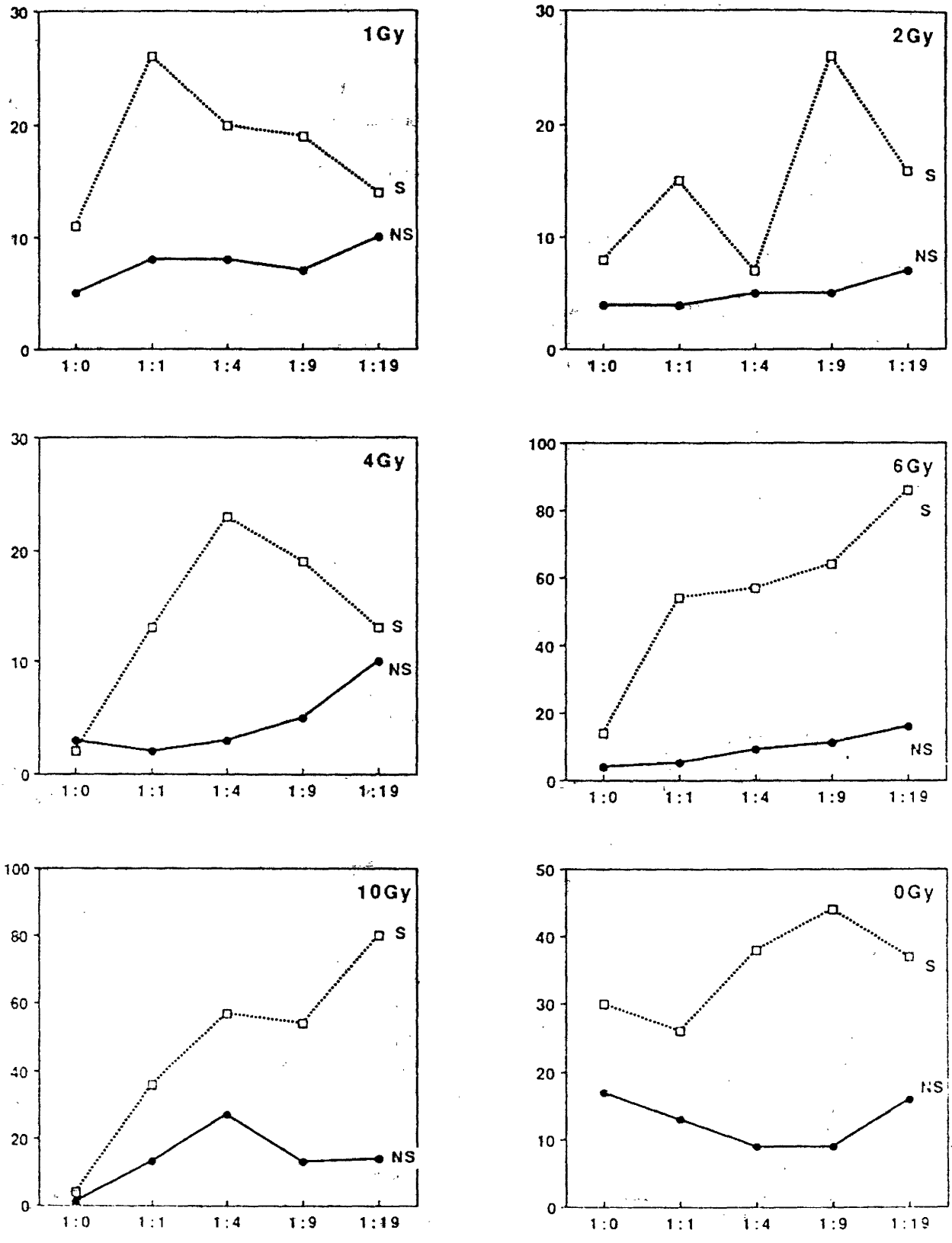


Fig. 1 : Mitotic index (ordinate) in relation to the dilution of irradiated cells from 1:0 to 1:19 (abscissa).
S = synchronized ; NS = non synchronized cultures. (TC 199 **medium**).

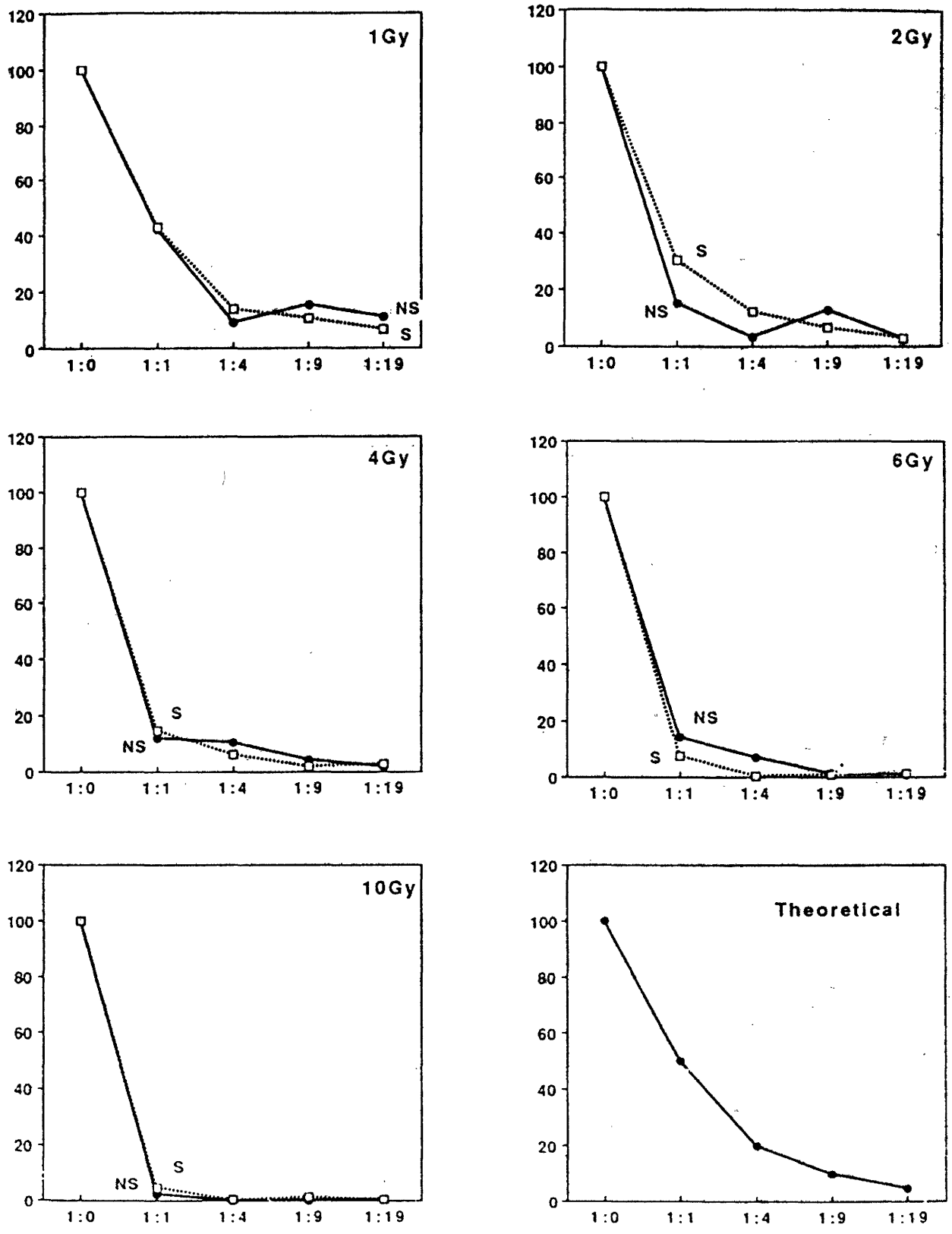
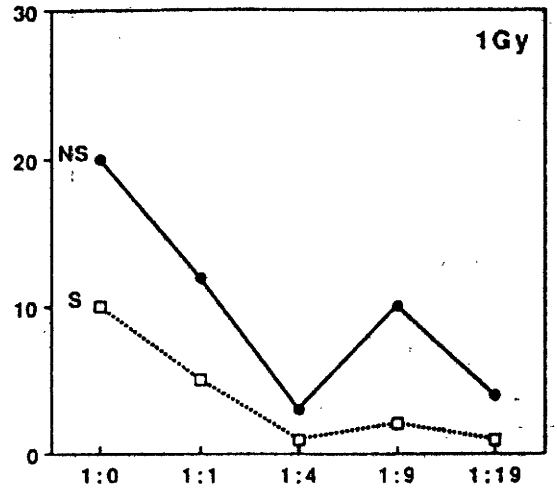
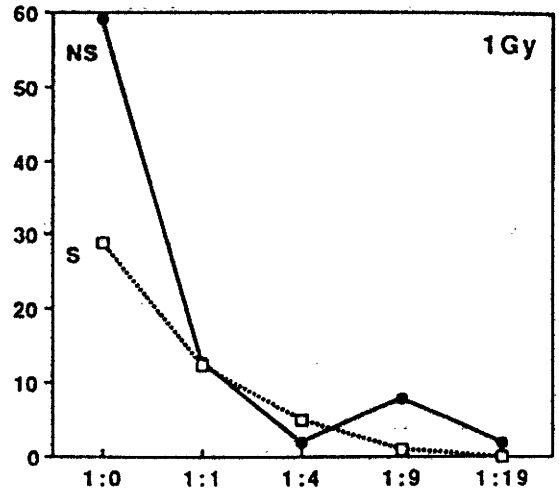
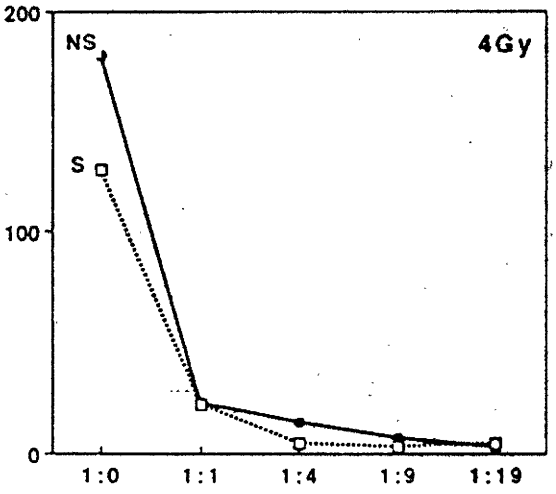
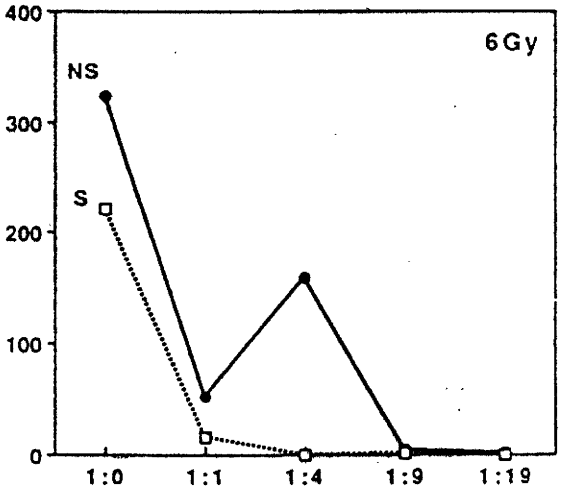


Fig. 2 : Percentages of male (irradiated) metaphases (ordinate) in relation to their dilution from 1:0 to 1:19 (abscissa).
S = synchronized, NS = non synchronized cultures. The theoretical graph is at the bottom, right. (TC 199 medium).









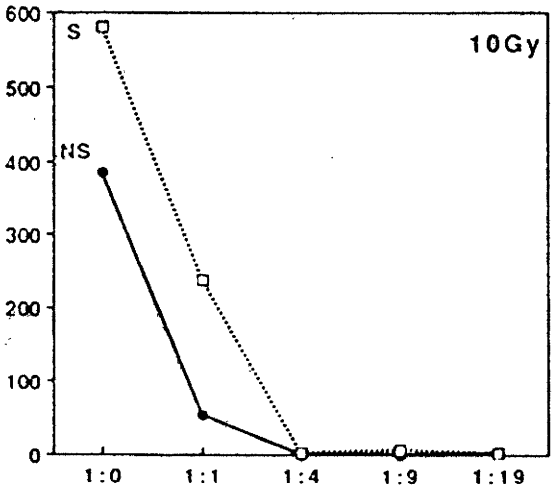
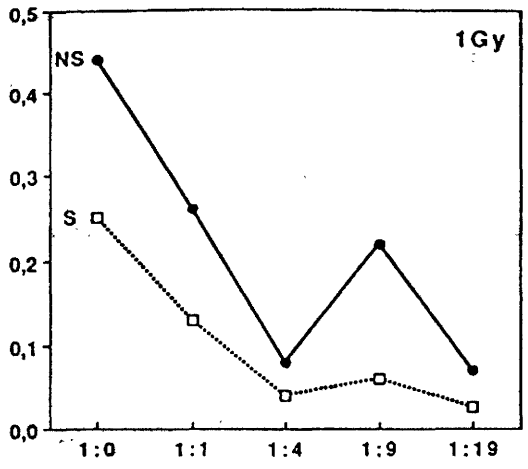
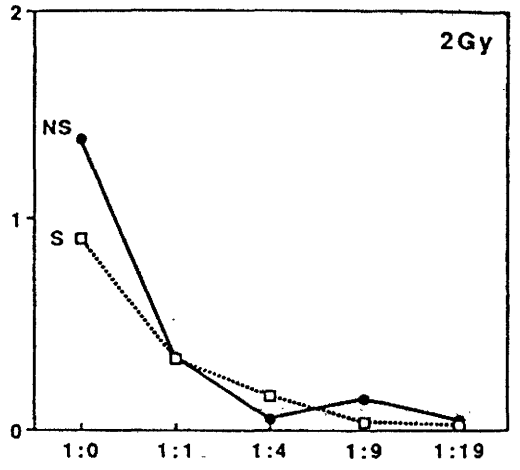
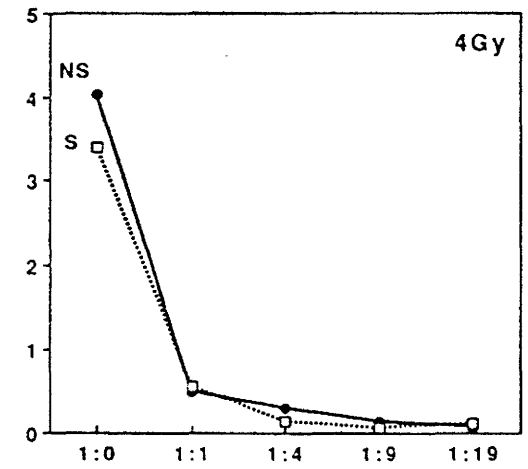
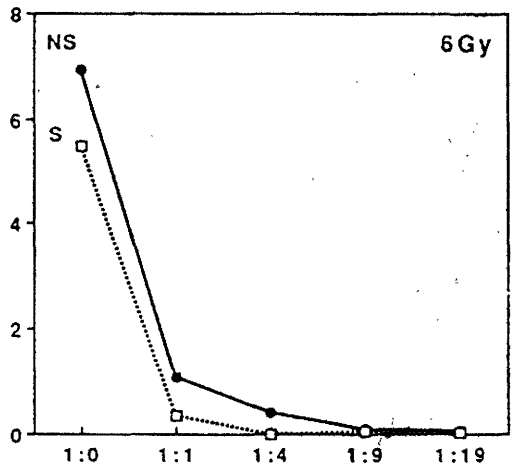


Fig. 3 : Average numbers of dicentrics plus rings (ordinate) per metaphase in relation to the dilution of irradiated cells (TC 199 medium).









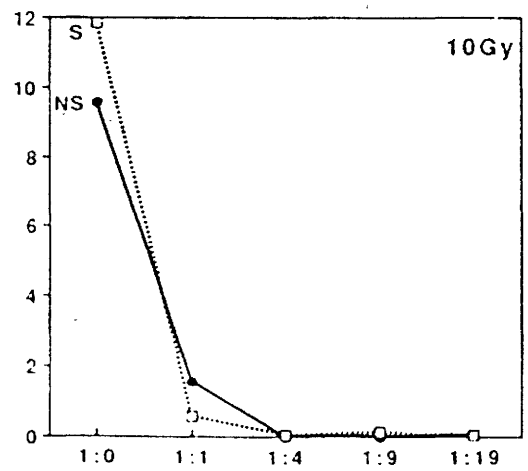


Fig. 4 : Average numbers of breaks, leading to dicentrics, rings and acentrics (ordinate) per metaphase, in relation to the dilution of irradiated cells (TC 199 medium).

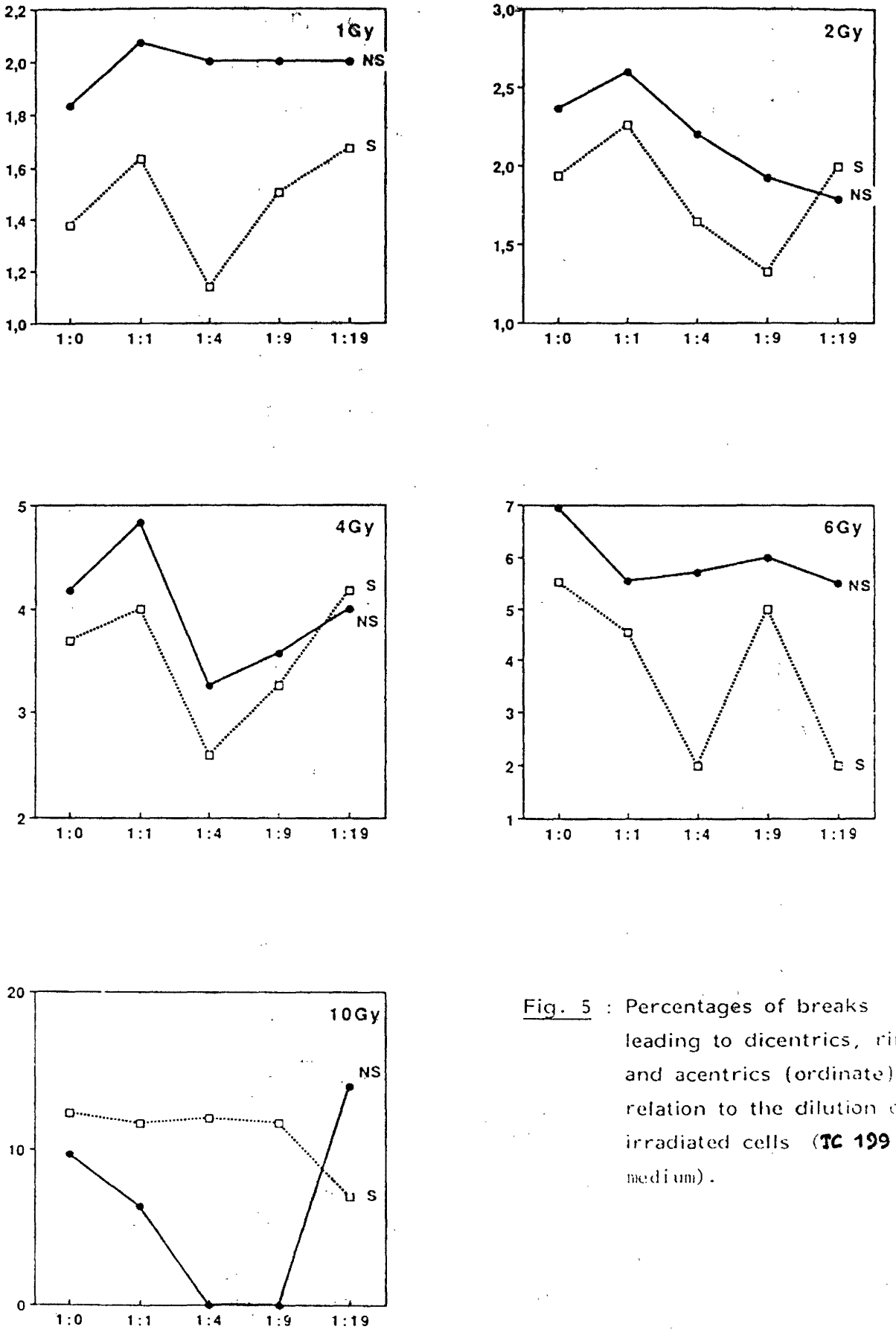


Fig. 5 : Percentages of breaks leading to dicentrics, rings and acentrics (ordinate) in relation to the dilution of irradiated cells (TC 199 medium).

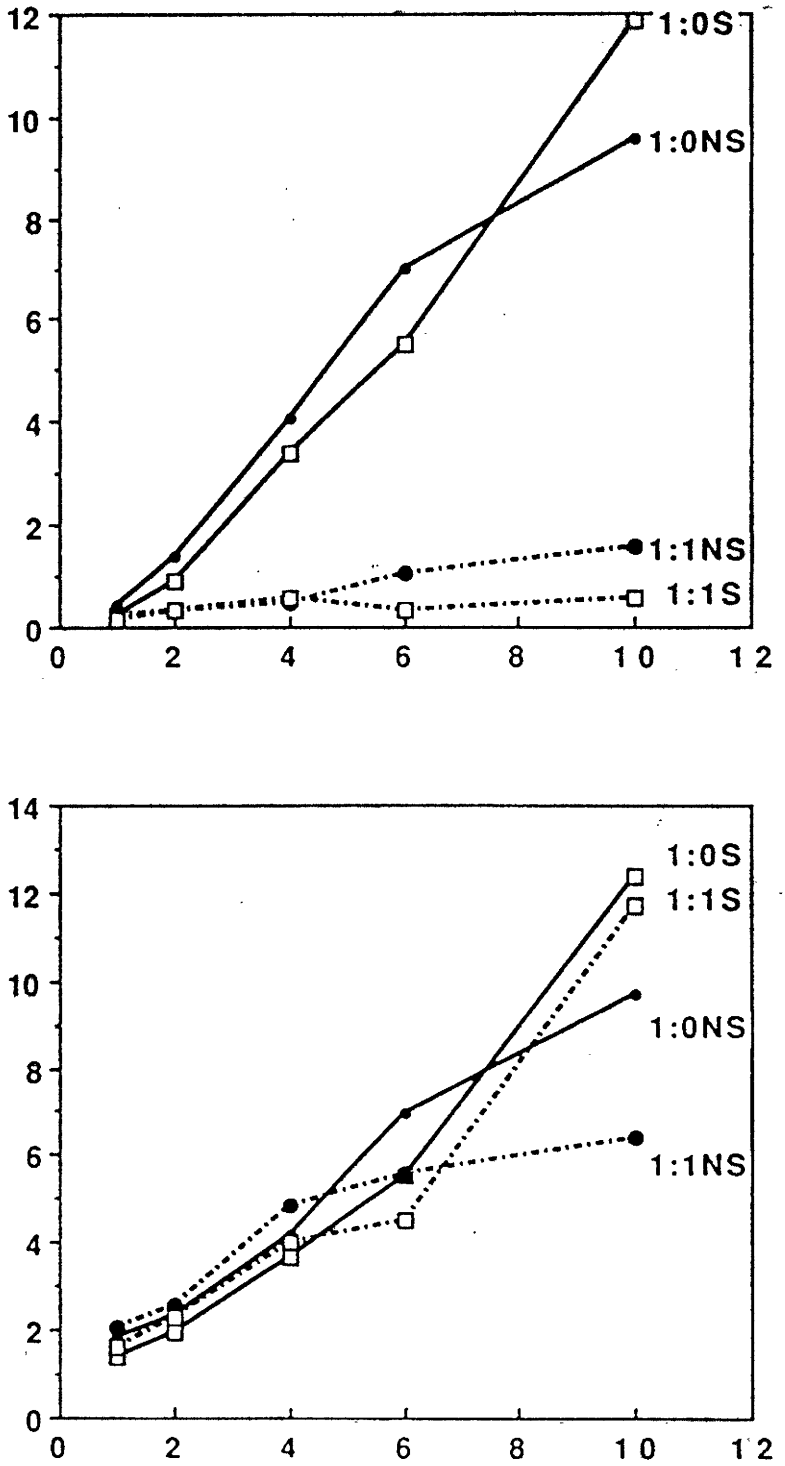


Fig. 6 : Percentages of breaks resulting in dicentrics, rings and acentrics (ordinate) in relation to the dose (1 to 10 Gy).
Top : 1:0 and 1:1 dilutions are compared, by the scoring of all metaphases. Bottom, the same but abnormal metaphases only were scored (TC 199 medium).

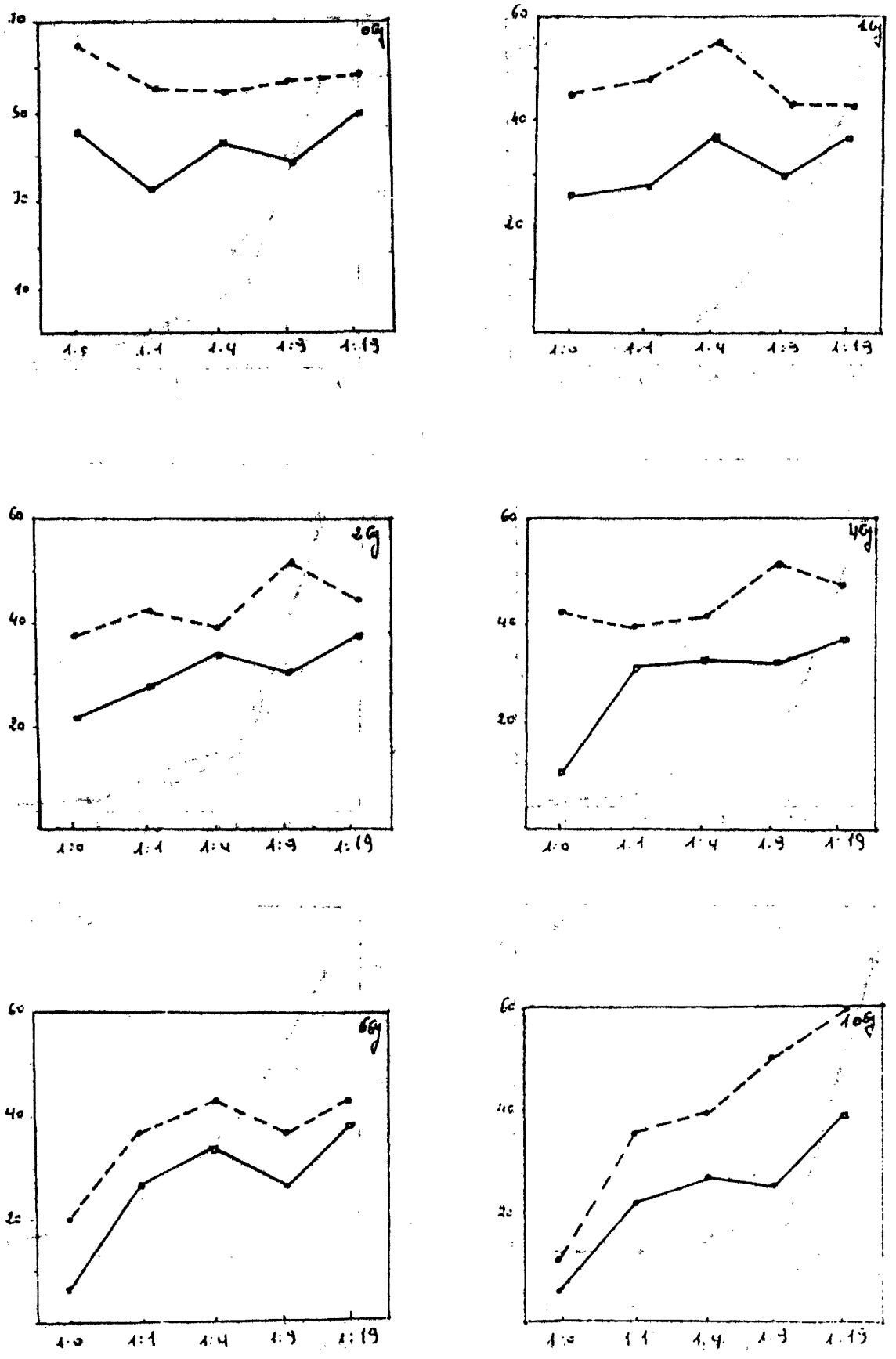


Fig.7 : Mitotic index (ordinate) in relation to the dilution of irradiated cells from 1.0 to 1.19 ---- with thymidine ; ---- without thymidine (Ham's medium)

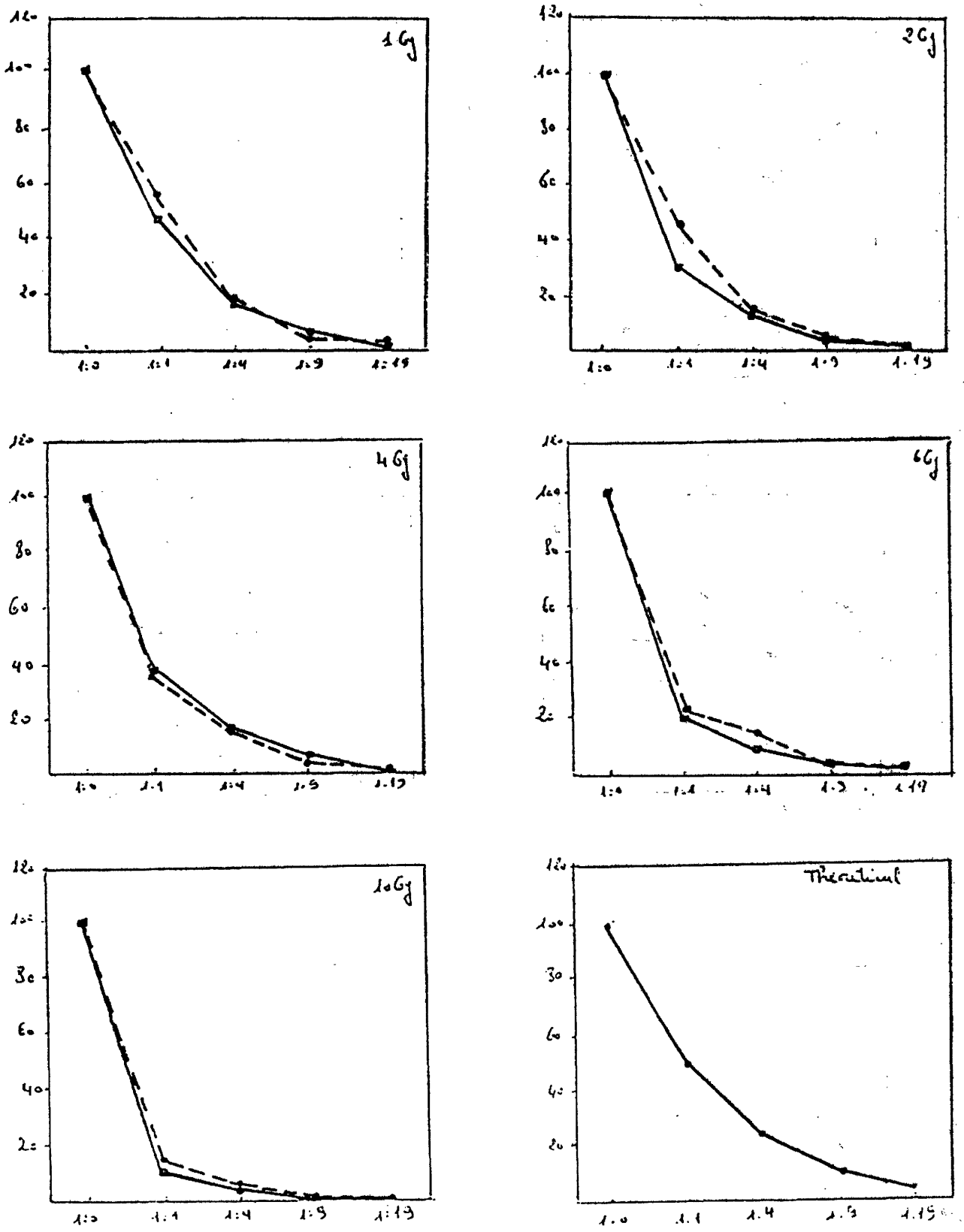
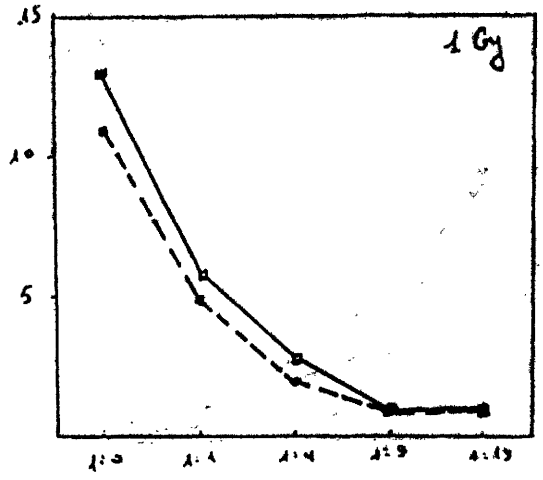


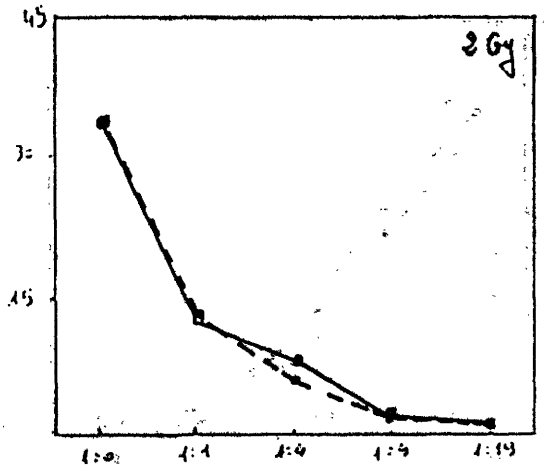
Fig.8 : Percentage of male (irradiated) metaphases (ordinate) in rhlation to their dilution from 1:0 to 1:19

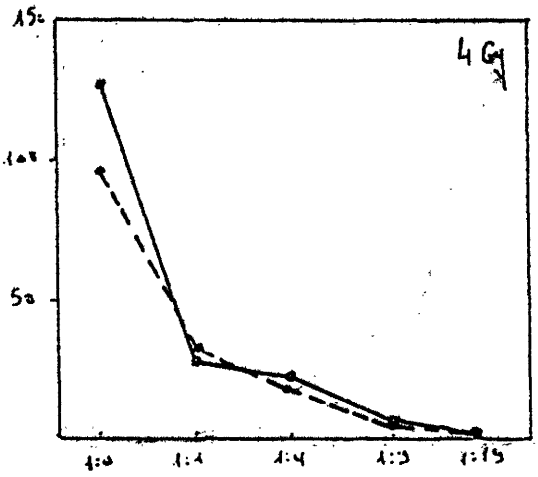
---- + Thymidine

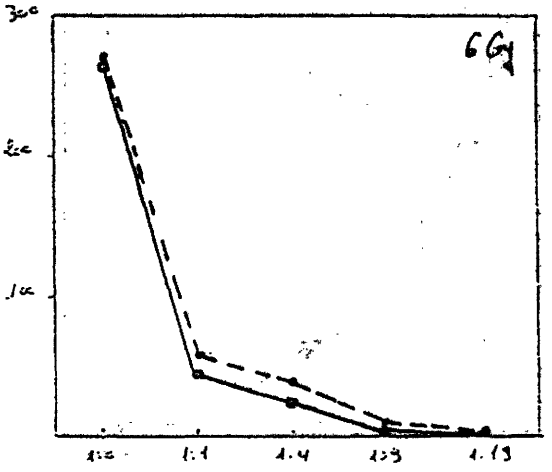
—— - Thymidine

(Ham's medium).









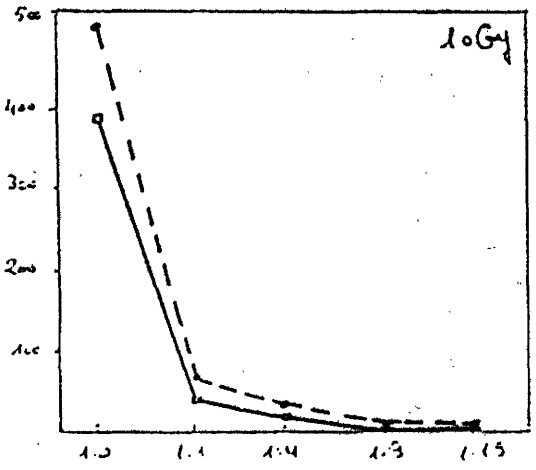


Fig.9 : Average number of dicentrics plus rings (ordinate) per 100 metaphases in relation to the dilution of irradiated cells

---- + Thymidine

----- - Thymidine

(Ham's medium)

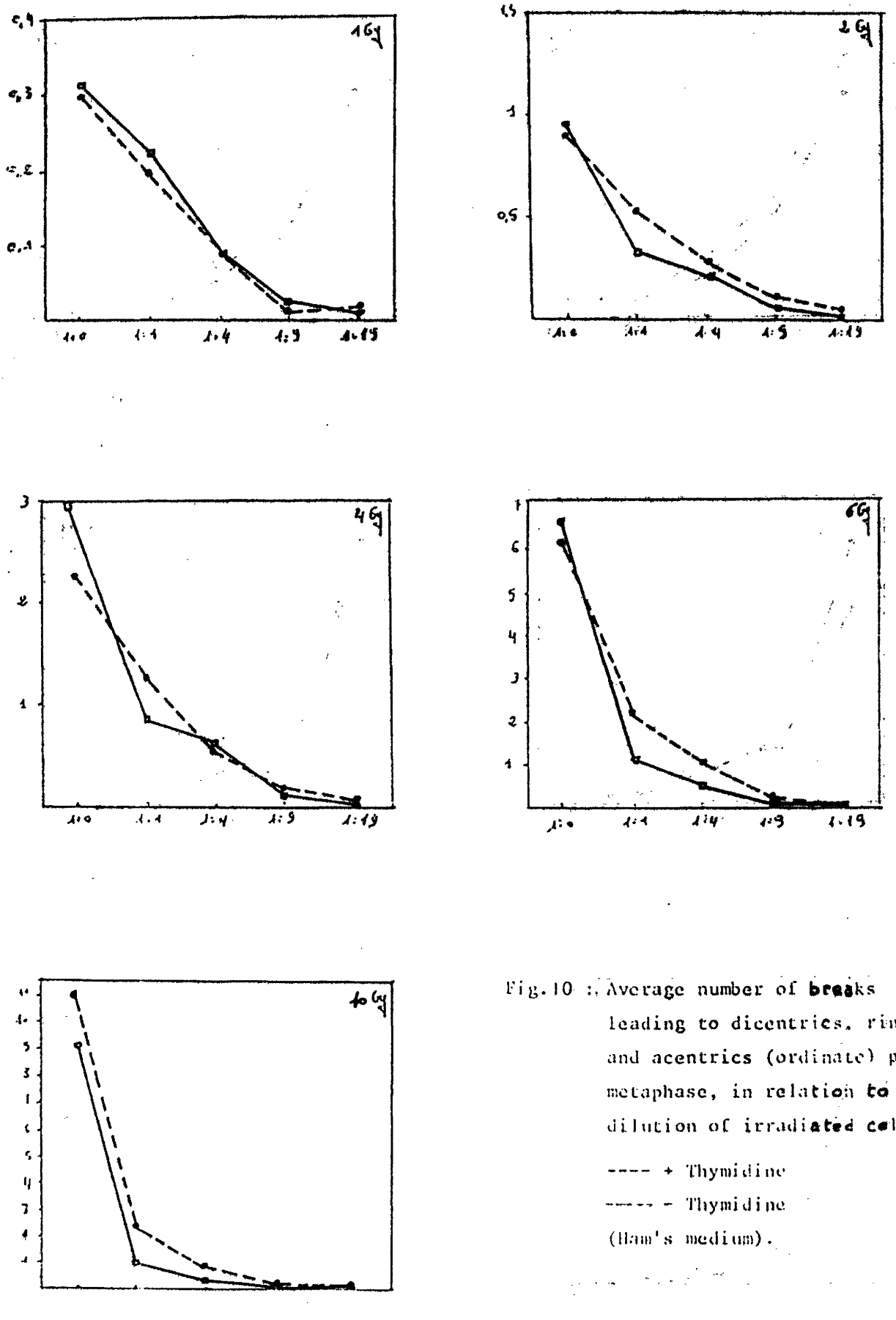


Fig.10 : Average number of breaks leading to dicentries, rings and acentrics (ordinate) per metaphase, in relation to the dilution of irradiated cells

---- + Thymidine

----- - Thymidine

(Ham's medium).

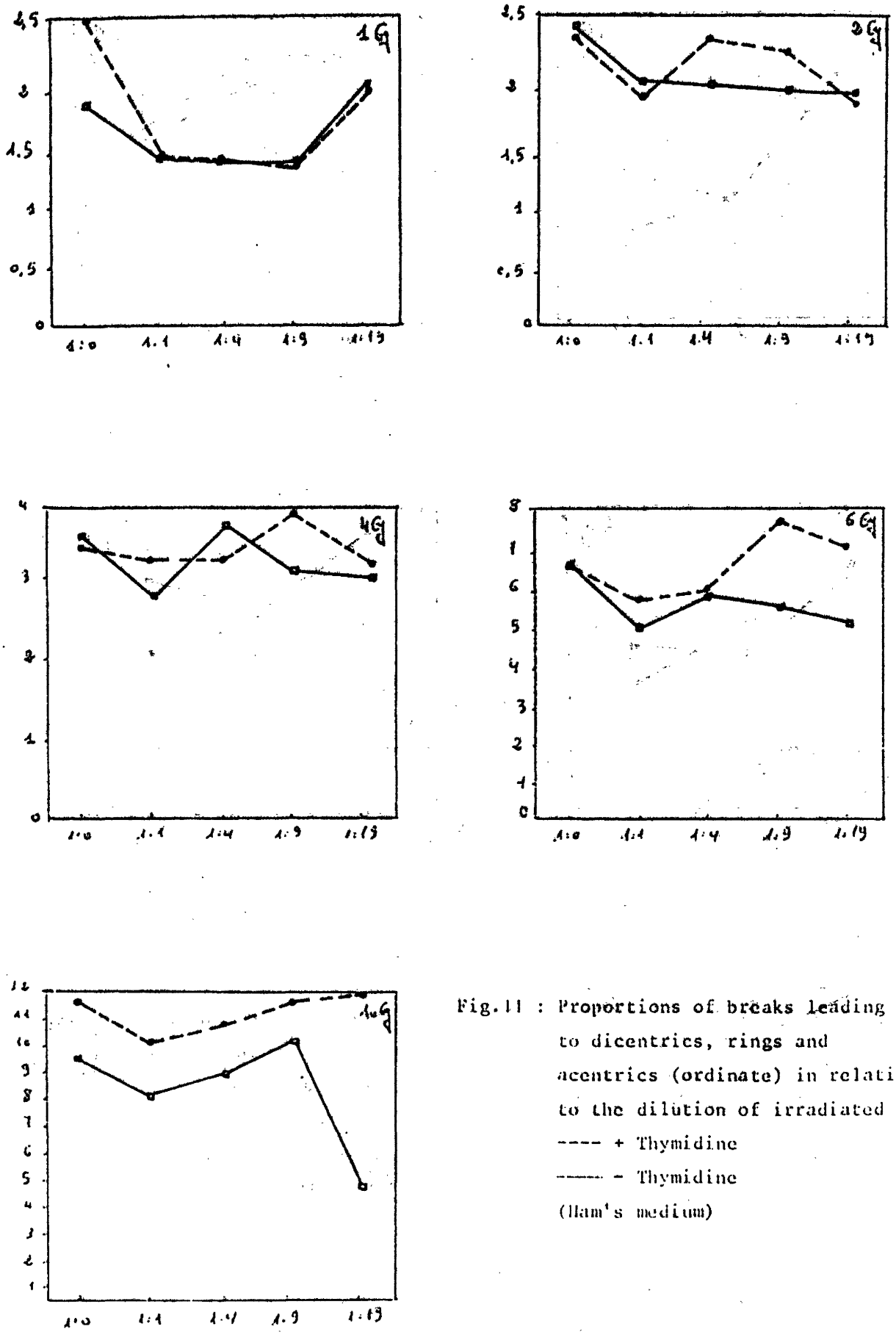


Fig.11 : Proportions of breaks leading to dicentrics, rings and acentrics (ordinate) in relation to the dilution of irradiated cells

---- + Thymidine

------ - Thymidine

(Ham's medium)

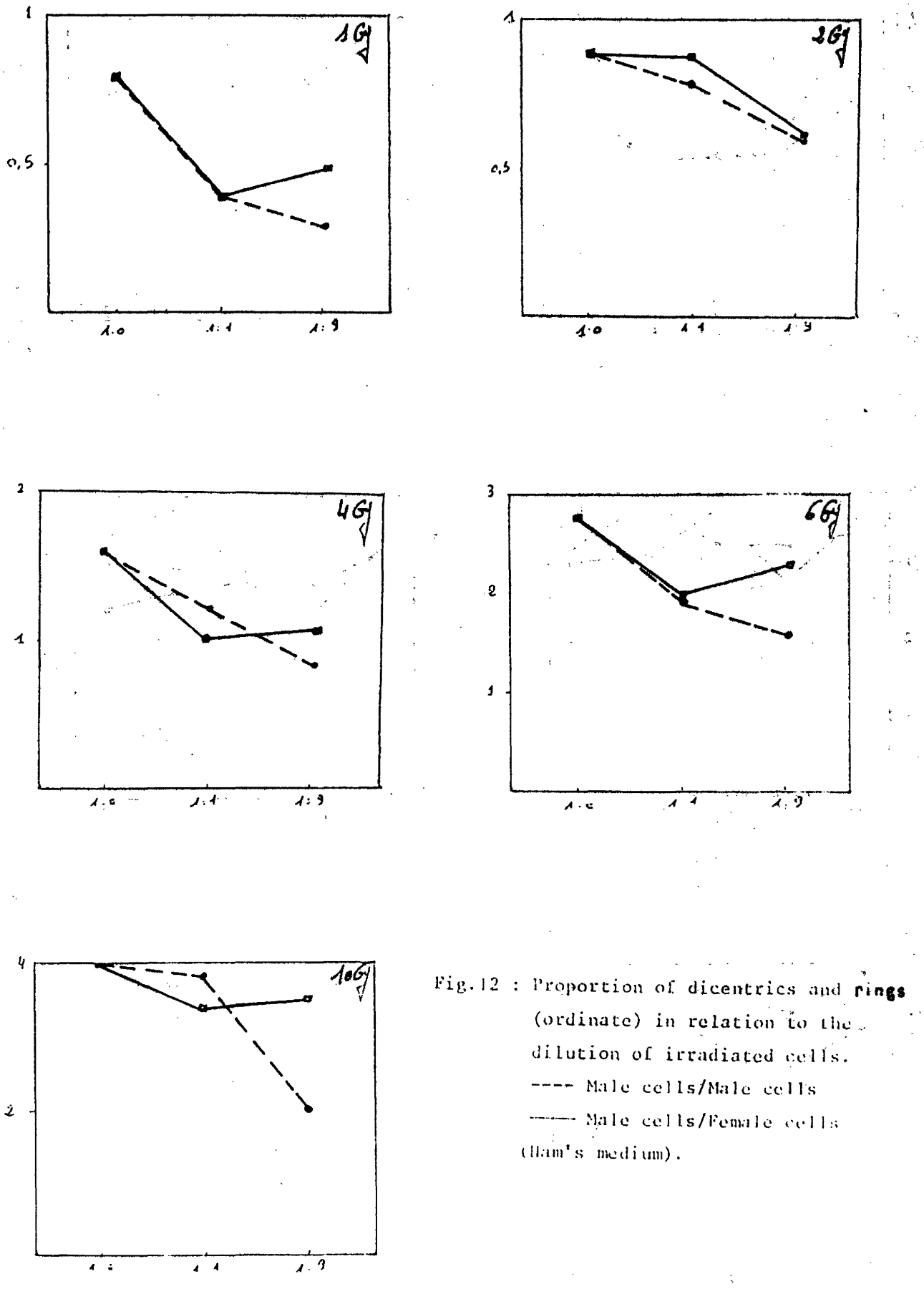


Fig.12 : Proportion of dicentrics and rings (ordinate) in relation to the dilution of irradiated cells.
---- Male cells/Male cells
—— Male cells/Female cells
(Ham's medium).

Dose to the male cells (Gy)	Male cells/ female cells	Cells scored	Male cells observed	Male cells with anomalies	Chromatid gap	Chromosome fragment	Translocation	Centric ring	Dicentric	Tricentric	Quadricentric
0	1:0	100	100	1	1						
	1:1	268	100	3	2	1					
	1:4	553	100	-	-	-					
	1:9	908	100	-	-	-					
	1:19	1022	43	2	1	1					
1	1:0	100	100	17	-	5		1	12		
	1:1	164	81	25	-	16		1	9		
	1:4	497	100	36	1	20		1	15		
	1:9	872	51	16	-	6	-	-	8		
	1:19	1093	33	7	-	2	-	-	6		
2	1:0	100	100	40		26	1	1	33		
	1:1	321	100	49	1	16	1	2	40		
	1:4	552	100	58	-	35	1	8	34		
	1:9	1531	69	43	2	26	-	7	23		
	1:19	937	27	16	1	8	-	-	9		
4	1:0	100	100	83	1	36	4	13	110	4	
	1:1	269	100	86	2	63	7	7	70	3	
	1:4	537	100	93	1	85	6	8	105	6	
	1:9	690	50	35	1	15	5	7	33	-	
	1:19	1121	28	21	1	11	1	4	20	1	
6	1:0	100	100	97	1	118	6	22	228	10	
	1:1	475	100	97	2	122	5	20	139	15	1
	1:4	958	100	99	2	97	11	30	195	8	-
	1:9	1162	44	37	2	33	4	6	68	4	1
	1:19	1234	19	19	-	13	4	4	34	1	-
10	1:0	100	100	96	-	148	12	44	274	26	6
	1:1	720	100	98	1	118	14	35	241	19	7
	1:4	1658	100	100	-	195	17	27	256	30	7
	1:9	1300	18	16	-	45	5	5	41	5	-
	1:19	1195	7	7	1	10	-	1	12	-	-

Table IX

Results of the cytogenetic observations (cultures without thymidine)

Dose to the male cells (Gy)	Male cells : female cells	Cells scored	Male cells observed	Male cells with anomalies	Chromatid gap	Chromosome fragment	Translocation	Centric ring	Dicentric	Tricentric	Quadricentric
0	1:0	100	100	1	1						
	1:1	230	100	–	–						
	1:4	448	100	–	–						
	1:9	1278	88	–	–						
	1:19	911	44	2	1	1					
1	1:0	100	100	13	–	11		3	8		
	1:1	172	100	33	3	26	1		9		
	1:4	470	100	33	5	24	–		11		
	1:9	1115	41	10	2	3	–		5		
	1:19	1123	39	12	–	11	1	1	4		
2	1:0	100	100	40	–	17	3	4	30		
	1:1	213	100	48	1	33	–	3	25	1	
	1:4	600	100	52	–	41	1	11	26		
	1:9	1044	60	33	1	17	2	3	23		
	1:19	982	35	22	–	16	1	1	12		
4	1:0	100	100	66	2	27	4	11	84	1	
	1:0	280	100	83	2	92	3	7	84	1	
	1:4	360	100	85	3	88	5	12	80	3	
	1:9	1147	53	47	3	46	8	10	49	1	
	1:19	1863	56	44	1	48	2	4	41	–	
6	1:0	100	100	90	–	67	8	40	200	14	2
	1:1	413	100	97	3	112	6	21	190	10	1
	1:4	588	100	98	–	118	7	23	198	13	1
	1:9	1366	49	49	1	58	13	21	119	6	–
	1:19	1650	27	26	1	36	5	10	57	2	1
10	1:0	100	100	93	–	134	6	46	370	30	7
	1:1	616	100	96	1	131	10	36	311	39	7
	1:4	1278	100	99	–	187	15	43	323	35	5
	1:9	1265	23	22	–	23	6	14	68	15	4
	1:19	1571	13	13	–	27	4	9	40	5	2

Table X

Results of the cytogenetic observations (cultures with thymidine)

Dose (Gy)	Male cells/ female cells	Dicentrics per abnormal cell	General mean
1	1:0	0.8	
	1:1	0.4	
	1:4	0.4	0.6
	1:9	0.5	
	1:19	0.9	
2	1:0	0.9	
	1:1	0.9	
	1:4	0.7	0.9
	1:9	0.7	
	1:19	0.7	
4	1:0	1.6	
	1:1	1.0	
	1:4	1.3	1.0
	1:9	1.1	
	1:19	1.2	
6	1:0	2.8	
	1:1	2.0	
	1:4	2.4	2.3
	1:9	2.3	
	1:19	2.1	
10	1:0	4.0	
	1:1	3.4	
	1:4	3.6	3.3
	1:9	3.5	
	1:19	3.9	

Table XI

Mean number of dicentrics per abnormal cell
(Culture without thymidine)

Dose (Gy)	Male cells/ female cells	Dicentrics per abnormal cell	General mean
1	1:0	0.8	
	1:1	0.3	
	1:4	0.3	0.5
	1:9	0.5	
	1:19	0.4	
2	1:0	0.9	
	1:1	0.6	
	1:4	0.7	0.7
	1:9	0.8	
	1:19	0.6	
4	1:0	1.5	
	1:1	1.1	
	1:4	1.2	1.2
	1:9	1.3	
	1:19	1.02	
6	1:0	3.04	
	1:1	2.4	
	1:4	2.6	2.8
	1:9	3.1	
	1:19	2.9	
10	1:0	5.3	
	1:1	4.7	
	1:4	4.6	5.04
	1:9	5.6	
	1:19	5	

Table XII

Mean number of dicentrics per abnormal cell
(Culture with thymidine)

Dose to the male cells (Gy)	Male cells/ female cells	Dicentrics + centric rings Expected (%)	Observed (%)	Difference
1	1:1	6	5	17 %
	1:4	3	2	33 %
	1:9	1	1	0
	1:19	1	1	0
2	1:1	17	14	18 %
	1:4	9	6	33 %
	1:9	3	2	33 %
	1:19	2	1	50 %
4	1:1	49	33	33 %
	1:4	24	18	25 %
	1:9	10	5	50 %
	1:19	5	2	60 %
6	1:1	137	57	58 %
	1:4	69	43	38 %
	1:9	27	11	59 %
	1:19	14	5	64 %
10	1:1	249	72	71 %
	1:4	124	35	72 %
	1:9	50	10	80 %
	1:19	25	4	84 %

Table XIII

Effect of the dilution and X-ray dose on the yield of dicentrics and centric rings in male dividing cells (Culture with thymidine in Ham's medium).

Dose to the male cells (Gy)	Male cells/ female cells	Dicentrics + Centric rings Expected (%)	Observed (%)	Difference
1	1:1	7	6	14 %
	1:4	3	3	-
	1:9	1	1	-
	1:19	1	1	-
2	1:1	17	13	24 %
	1:4	9	8	11 %
	1:9	3	2	33 %
	1:19	2	1	50 %
4	1:1	66	31	53 %
	1:4	33	23	30 %
	1:9	13	6	54 %
	1:19	7	2	71 %
6	1:1	135	40	70 %
	1:4	68	25	63 %
	1:9	27	7	74 %
	1:19	14	3	79 %
10	1:1	194	47	76 %
	1:4	97	22	77 %
	1:9	39	4	90 %
	1:19	19	1	95 %

Table XIV

Effect of the dilution and X-ray dose on the yield of dicentrics and centric rings in male dividing cells (culture without thymidine in Ham's medium).

Male cells/ female cells	Dose to the male cells (Gy)	Cells scored	Total number of dicentrics	Number of dicentrics + rings per cell												d	u
				0	1	2	3	4	5	6	7	8	9	10	11		
1:0	1	100	13	88	11	1										1,02	0,2
	2	100	34	72	22	6										1,01	0,1
	4	100	131	26	33	28	10	3								0,9	- 1,1
	6	100	270	5	15	29	22	18	9	2						0,7	- 1,9
	10	100	388	4	2	16	18	22	20	10	8					0,8	- 1,9
1:0	1	164	10	154	10											0,9	- 0,6
	2	321	42	284	32	5										1,1	1,4
	4	269	83	210	42	11	5	1								1,5	5,4**
	6	475	192	388	34	23	16	9	3	1	1					2,6	24,3**
	10	720	335	631	12	12	16	25	8	6	8	1	0	0	1	4,3	62,7**
1:4	1	497	16	481	16											0,9	- 0,5
	2	552	42	518	26	8										1,3	5,1**
	4	537	125	460	44	26	3	2	0	2						1,9	16,4**
	6	958	241	864	22	28	22	13	9							2,9	42,2**
	10	1658	364	1566	6	7	20	27	19	11	1	1				4,3	93,9**
1:9	1	872	8	829	8											0,9	- 0,2
	2	1531	30	1506	20	5										1,3	8,8**
	4	690	40	662	19	6	3									1,7	13,1**
	6	1162	85	1128	7	12	9	4	1	1						2,9	48,4**
	10	1300	56	1284	1	3	3	6	2	1						3,9	75,1**
1:19	1	1093	6	1087	6											0,9	- 0,1
	2	937	9	928	9											0,9	- 0,2
	4	1121	26	1104	10	5	2									1,8	19,9**
	6	1234	40	1217	4	6	4	3								2,8	44,5**
	10	1194	13	1188	3	2	2									2,2	31**

Table XV

Distribution of dicentrics and centric rings in pure and mixed blood after exposure to X-rays.
(Culture without thymidine in Ham's medium).
The U test (Papworth, 1970) was used for statistical analysis
(x = $p < 0,05$; xx = $P < 0,01$).

Male cells/ female cells	Dose to the male cells (Gy)	Cells scored	Total number of dicentric	Number of dicentrics + rings per cell													d	u
				0	1	2	3	4	5	6	7	8	9	10	11	12		
1:0	1	100	11	89	11												0,8	- 0,8
	2	100	34	71	24	5											0,9	- 0,3
	4	100	97	38	38	16	6	1	1								1,1	0,4
	6	100	274	10	12	23	24	16	12	1	2						0,9	- 0,3
	10	100	497	10	6	1	6	19	16	11	15	1	4	2	0	2	1,5	3,6^{xx}
1:1	1	172	9	163	9												0,9	- 0,5
	2	213	30	188	21	3	1										1,3	2,7^{x}
	4	280	93	226	27	16	10	1									1,8	9,3^{xx}
	6	413	234	320	23	28	23	12	4	3							2,6	23,1^{xx}
	10	616	446	519	1	10	17	16	26	13	11	2	1				4,5	60,9^{xx}
1:4	1	470	11	459	11												0,9	- 0,4
	2	600	37	568	27	5											1,2	3,7^{xx}
	4	560	98	494	41	18	7										1,6	10,4^{xx}
	6	588	250	494	21	25	26	12	7	3							2,9	32,5^{xx}
	10	1278	451	1182	5	6	15	25	16	11	9	4	2	3			5,2	107^{xx}
1:9	1	1115	5	1110	5												0,9	0,1
	2	1044	26	1023	16	5											1,4	8,4^{xx}
	4	1147	61	1110	23	6	6	2									2,1	27,2^{xx}
	6	1366	152	1318	4	14	15	9	1	2	2	1					3,8	73,9^{xx}
	10	1265	124	1243	1	0	1	4	5	4	3	2	2				6,2^{x}	131,1^{xx}
1:19	1	1123	5	1118	5												0,9	- 0,1
	2	982	13	969	13												0,9	- 0,3
	4	1863	45	1835	16	7	5										1,95	- 0,3
	6	1650	74	1624	6	3	8	7	2								3,4	68,2^{xx}
	10	1571	65	1558	1	1	0	4	4	0	1	1	0	0	1		6,2	145,7^{xx}

Table XVI

Distribution of dicentrics and centric rings in pure and mixed blood after exposure to X-rays (Culture with thymidine in Ham's medium).
The U test (Papworth, 1979) was used for statistical analysis.
($x = p < 0,05$; $xx = p < 0,01$).

Table XVII

Dicentric aberrations and estimate of irradiated fraction after 1 Gy administrated to male cells mixed in various proportions to unirradiated female cells (Cultures in Ham's medium without thymidine).

Fraction irradiated	Cells scored	% of cells with dicentrics and/or centric rings	Observed dicenttic mean in cells with dicentrics	Maximum dicentric number observed in damaged cells	Fraction estimate
1	100	12	1.1	2	-
0.5	164	6	1	1	-
0.2	497	3	1	1	-
0.10	872	1	1	1	-
0.05	1093	1	1	1	-

Table XVIII

Dicentric aberrations and estimate of irradiated fraction after 2 Gy administrated to male cells mixed in various proportions to unirradiated female cells (Cultures in Ham's medium without thymidine).

Fraction irradiated	Cells scored	% of cells with dicentrics and/or centric rings	Observed dicentric mean in cells with dicentrics	Maximum dicentric number observed in damaged cells	Fraction estimate
1	100	28	1.2	2	-
0.5	321	12	1.1	2	0.6
0.2	552	6	1.2	2	0.28
0.10	1531	2	1.2	2	0.09
0.05	937	1	1	1	-

Table XIX

Dicentric aberrations and estimate of irradiated fraction after 4 Gy administrated to male cells mixed in various proportions to unirradiated female cells.
(Cultures in Ham's medium without thymidine).

Fraction irradiated	Cells scored	% of cells with dicentrics and/or centric rings	Observed dicentric mean in cells with dicentrics	Maximum dicentric number observed in damaged cells	Fraction estimate
1	100	74	1.8	4	-
0.5	269	22	1.4	4	0.6
0.2	537	14	1.6	6	0.5
0.10	690	4	1.4	3	0.2
0.05	1121	2	1.5	3	0.07

Table XX

Dicentric aberrations and estimate of irradiated fraction after 6 Gy administrated to male cells mixed in various proportions to unirradiated female cells.
(Cultures in Ham's medium without thymidine).

Fraction irradiated	Cells scored	% of cells with dicentries and/or centric rings	Observed dicentric mean in cells with dicentrics	Maximum dicentric number observed in damaged cells	Fraction estimate
1	100	95	2.8	6	-
0.5	475	19	2.2	7	0.6
0.2	958	10	2.6	5	0.5
0.10	1162	3	2.5	6	0.2
0.05	1234	1	2.4	4	0.1

Table XXI

Dicentric aberrations and estimate of irradiated fraction after 10 Gy administrated to male cells mixed in various proportions to unirradiated female cells.
(Cultures in Ham's medium without thymidine).

Fraction irradiated	Cells scored	% of cells with dicentries and/or centric rings	Observed dicentric mean in cells with dicentrics	Maximum dicentric number observed in damaged cells	Fraction estimate
1	100	96	4.1	7	-
0.5	720	12	3.8	11	0.7
0.2	1658	6	4	8	0.6
0.10	1300	1	3.5	6	0.2
0.05	1195	1	1.9	3	0.04

Table XXII

Dicentric aberrations and estimate of irradiated fraction after 1 Gy administrated to male cells mixed in various proportions to unirradiated female cells.
(Cultures in Ham's medium with thymidine).

Fraction irradiated	Cells scored	% of cells with dicentrics and/or centric rings	Observed dicentric mean in cells with dicentrics	Maximum dicentric number observed in damaged cells	Fraction estimate
1	100	11	1	1	-
0.5	172	5	1	1	0.2
0.2	470	2	1	1	-
0.10	1115	1	1	1	-
0.05	1123	1	1	1	-

Table XXIII

Dicentric aberrations and estimate of irradiated fraction after 2 Gy administrated to male cells mixed in various proportions to unirradiated female cells.
(Cultures in Ham's medium with thymidine).

Fraction irradiated	Cells scored	% of cells with dicentrics and/or centric rings	Observed dicentric mean in cells with dicentrics	Maximum dicentric number observed in damaged cells	Fraction estimate
1	100	29	1.2	2	-
0.5	213	12	1.2	3	0.5
0.2	600	5	1.2	2	0.3
0.10	1044	2	1.2	2	0.1
0.05	982	1	1	1	-

Table XXIV

Dicentric aberrations and estimate of irradiated fraction after 4 Gy administrated to male cells mixed in various proportions to unirradiated female cells.
(Cultures in Ham's medium with thymidine).

Fraction irradiated	Cells socred	% of cells with dicentrics and/or centric rings	Observed dicentric mean in cells with dicentrics	Maximum dicentric number observed in damaged cells	Fraction estimate
1	100	62	1.6	5	-
0.5	289	19	1.7	4	0.6
0.2	560	12	1.5	3	0.4
0.10	1147	3	1.6	4	0.2
0.05	1863	2	1.6	3	0.09

Table XXV

Dicentric aberrations and estimate of irradiated fraction after 6 Gy administrated to male cells mixed in various proportions to unirradiated female cells.
(Cultures in Ham's medium with thymidine).

Fraction irradiated	Cells scored	% of cells with dicentrics and/or centric rings	Observed dicentric mean in cells with dicentrics	Maximum dicentric number observed in damaged cells	Fraction estimate
1	100	90	3	7	-
0.5	413	23	2.5	6	0.6
0.2	588	16	2.7	6	0.6
0.10	1366	4	3.2	8	0.3
0.05	1650	2	2.8	5	0.1

Table XXVI

Dicentric aberrations and estimate of irradiated fraction after 10 Gy administrated to male cells mixed in various proportions to unirradiated female cells.
(Cultures in Ham's medium with thymidine).

Fraction irradiated	Cells scored	% of cells with dicentrics and/or centric rings	Observed dicentric mean in cells with dicentrics	Maximum dicentric number observed in	Fraction estimate
1	100	90	5.5	12	-
0.5	616	16	4.6	9	0.8
0.2	1278	8	4.7	10	0.7
0.10	1265	2	5.6	9	0.4
0.05	1571	1	5	11	0.2

Table XXVII

Estimation of the dose for cells treated with and without thymidine by the contaminated Poisson.
(Cultures in Ham's medium).

Doses administrated (Gy)	Dilution	Doses estimated (Gy)	
		With thymidine	Without thymidine
2	1:1	2.01	1.10
	1:4	1.71	1.75
	1:9	2.24	1.53
	1:19	0.02	0
4	1:1	4.24	2.68
	1:4	3.42	3.63
	1:9	4.01	2.78
	1:19	3.87	3.84
6	1:1	6.40	5.56
	1:4	6.40	6.49
	1:9	7.25	6.33
	1:19	6.73	5.95
10	1:1	9.14	8.93
	1:4	9.26	9.27
	1:9	10.27	8.46
	1:19	9.60	5.44

NATIONAL RADIOLOGICAL PROTECTION BOARD

UNIVERSITEIT LEIDEN

LAB. STRAALENGENETICA EN CHEMISCHE MUTAGENESE

CONTRACT BI6 - 0286 - UK
CONTRACT BI6 - 0287 - NL

BILOGICAL DOSIMETRY APPLIED TO IN VITRO SIMULATED PARTIAL BODY IRRADIATION

D. C. LLOYD, A. A. EDWARDS, A. T. NATARAJAN, F. DARROUDI.

Introduction

The scientific and medical response to an accidental overexposure to ionising radiation should include an attempt to estimate absorbed doses to irradiated persons. This information may be gained from physical and biological methods, although in some situations, such as the Chernobyl accident, information from physical techniques may be scant. At Chernobyl the initial sorting of casualties was done by observations of the speed of onset and severity of the prodromal response. More precise biological dosimetry was then performed using the well established technique of analysing peripheral blood lymphocytes for dicentric chromosomal aberrations. In the event of another Chernobyl-like accident, chromosomal studies would again be the method of choice for biological dosimetry. One could envisage studies needing to be carried out on perhaps a few hundred subjects exhibiting prodromal reactions and several hundreds more possibly exposed to doses below the threshold for such early symptoms (1 Gy). As chromosomal analysis is particularly labour intensive and time consuming, there may be considerable logistical problems in dealing with large numbers of patients. The newer micronucleus assay that utilises cytochalsin B to block cytokinesis offers an alternative to scoring for dicentrics. Because the images are far simpler, the analysis time could be completed significantly faster and by less skilled technicians. There is usually an urgent need to provide an estimate of dose as quickly as possible and present techniques are not ideal in this respect because of the need to culture lymphocytes for two days for chromosomal aberrations or three days for the micronucleus method. The method of prematurely condensed chromosomes (PCC) however, opens up the possibility of scoring aberrations within a few hours of blood sampling.

Accidental irradiation is usually inhomogeneous. For highly irradiated patients the clinician requires information on the inhomogeneity of the exposure as management of casualties may require an early decision on whether

treatment should include transplantation of allogenic bone marrow or treatment with growth factors. If there is a likelihood that some of the patient's own marrow will have survived because of inhomogeneous exposure, then transplantation should be avoided. Graft rejection was observed in several of the Chernobyl patients (Baranov et al 1989) and in some cases probably contributed to their deaths.

Two methods have been proposed for analysing chromosomal aberration data from inhomogeneously exposed persons with the objective of improving the dose estimates to reflect non-uniformity, rather than quoting an averaged whole body dose estimate that is obtained simply from relating a dicentric yield to an in vitro dose response curve. The first method proposed by Dolphin (1969) considered the over dispersed distribution of aberrations, particularly dicentrics, among the scored cells. The distribution is considered to be the sum of a Poisson distribution which represents the irradiated fraction of the body and the remaining unexposed fraction. Cells containing aberrations will obviously have been in the irradiated part of the body. Normal undamaged cells will comprise two sub-populations, those from the unexposed fraction and irradiated cells representing the first term of the Poisson series. This assumption enables one to go on to derive, by a procedure that is discussed more fully in the Appendix to this paper, an estimate of the size of the irradiated fraction and its mean dose. Previously published data from in vitro simulation of half-body irradiations have shown that this procedure is viable but of necessity requires a number of simplifying assumptions concerning the distribution of lymphocytes throughout the body (Lloyd et al 1987, IAEA 1986). Estimates of blood volume exposed may not closely reflect the proportion of body mass exposed (Liniecki et al 1983).

The second approach termed the Qdr method was proposed by Sasaki and Miyata (1968). This considers the yield of dicentrics and rings only from those cells that contain unstable aberrations and were therefore in the

irradiated part of the body. The method circumvents the problem of dilution by undamaged cells from an unexposed part. Qdr is a function of dose alone and so permits a dose estimate to be made for the irradiated part of the body. As originally published the method did not go on to determine the size of the irradiated fraction but Sasaki (1983) has later shown that this can be estimated using the same principle as for the contaminated Poisson technique.

The objective of the present work is to simulate in vitro high doses that are life threatening if they involve the whole body. If however, they are received by only part of the body and good medical care is provided there are hopes for survival. Different ratios of irradiated to unirradiated fractions are simulated and the limits of resolution for detecting this by chromosomal aberration, micronucleus and PCC endpoints are investigated.

Materials and Methods

Blood samples were taken in Chilton from two healthy non-smoking male donors aged 24 and 46. The blood was placed into a number of lithium heparin Vacutainer tubes kept at 37°C and some tubes were irradiated to 5 or 8 Gy with 250 kVp X-rays HVL 1.22mm Cu at 1Gy per min. One donor's blood was used for each dose. In order to obtain sufficient volumes of blood at each dose, several tubes were irradiated placed side by side. After exposure the blood from the replicate tubes was allowed to fall to room temperature and pooled into glass containers so that any slight variations in dose between replicates was equalised. Uncertainties in the stated doses are $\pm$ 1.5%. The unirradiated blood taken at the same time was treated similarly. For each dose volumes of irradiated and unirradiated blood from the same donor were mixed in differing proportions in order to simulate the mixing of blood that would occur after acute partial body irradiation. The irradiated fractions range from 99% to 10% and are shown in Tables 1-5.

The samples were coded for blind analysis and shared between Chilton and Leiden. The Leiden specimens were sent by a commerical courier service and

arrived during the following day. The Chilton specimens were kept at room temperature for 24 h before cultures were set up so that the blood processed in each laboratory was subject to approximately the same delay after irradiation. In Chilton the blood was cultured for conventional metaphase aberrations and for cytokinesis blocked micronucleus preparations. In Leiden aberrations and micronucleus cultures were also set up and in addition preparations for PCC were made.

Aberrations

Standard 48 hour whole blood cultures were set up and processed following the method described in an IAEA manual (1986). In Chilton the medium used was Eagles MEM + 10% fetal calf serum and in Leiden F10 medium plus 20% fetal calf serum. At each dose and mixture, several replicate cultures were made. The fixed cells from the replicates were pooled before dropping onto slides. Bromodeoxyuridine was included in the culture medium so that replicate slides from each point were checked by fluorescence plus Giemsa staining to ensure that the number of second division cells was low (<5% and frequently zero). Having established that second division contaminations were low, the remaining slides were stained with conventional Giemsa and scored for unstable chromosomal aberrations; dicentrics, centric rings and excess acentrics.

Micronuclei

Seventy two hour cultures for assaying micronuclei in cytokinesis blocked lymphocytes were made and, as described above, fixed replicates were pooled before placing the cells onto slides. The procedure followed the general method described by Fenech and Morley (1985). The exact techniques and scoring criteria used at Chilton and Leiden are given in Prosser et al (1988) and Ramalho et al (1988) respectively. Binucleate cells stained with Giemsa were scored for the presence of micronuclei.

Prematurely condensed chromosomes (PCC)

In Leiden, Ficoll separated lymphocytes were fused with mitotic Chinese hamster cells by the polyethyleneglycol method (Pantelias and Maillie 1983).

The lymphocytes were fixed one hour after fusion according to the standard protocol. The exact procedure used in Leiden is described by Natarajan et al (1986). The clusters of single stranded human chromosomes condensed in G_o were examined and those comprising 46 or more objects were recorded.

Results and Discussion

The measured yields of chromosomal aberrations in 48 hour lymphocyte cultures observed by the two laboratories at the two doses are shown in Tables 1 and 2. The results for micronuclei are shown in Tables 3 and 4 and for PCC in Table 5.

Unstable chromosomal aberrations

Table 6 shows an analysis of the dicentric distributions from Chilton to derive both the fraction irradiated and its dose compared with the known values for each mixture. The methods used were the contaminated Poisson method as described in the Appendix and the Qdr method or more strictly the Qd method as it has been applied to the dicentrics alone rather than the dicentrics and rings as originally formulated. The calculations of estimated dose utilised dose response curves previously published (Lloyd et al 1986) by Chilton for dicentrics

$$Y = ((3.64 \pm 0.53)10^{-2}D + (6.67 \pm 0.22)10^{-2}D^2) \qquad ...(1)$$

and for total aberrations

$$Y = ((5.10 \pm 1.69)10^{-2}D + (11.1 \pm 0.7)10^{-2}D^2) \qquad ...(2)$$

For calculating the fraction irradiated from the fraction irradiated and seen (see equation 13 in Appendix) a D_o value of 2.5 Gy was used. The true doses and actual irradiated fractions as shown in columns 1 and 2, are very well estimated by both methods. At both doses the mean of doses in column 4 are within 10% of the known values. The discrepancy is a little higher at 8 Gy possibly because at this dose the effects of saturation may been seen. A test for constancy of the estimates of dose to the irradiated fraction shows a χ^2 value a little greater than the degrees of freedom. The data points which

deviate from the known dose and fraction irradiated by more than two standard errors are indicated in Table 6. There is very little difference between the corresponding estimates from the two methods. For the Qd method no estimates of uncertainties are given because Sasaki does not give the method of deriving these and the analysis is not a trivial one. However, standard errors should not be too different from those given for the contaminated Poisson method.

Leiden chose to combine dicentrics and centric rings and in Table 7 the results are analysed. Because the yield for the 100% irradiated sample differed from those of Chilton, it was not possible to use the yield curve (eq 1) shown above as applied in Table 6 to the data from Chilton. Therefore, estimates of dose to the irradiated fraction could not be derived for Leiden. Neither was it possible to use the Qdr method because a yield curve for total unstable aberrations is required. Leiden has not generated its own dose response data for this quality of X-rays. The present data were therefore analysed using the contaminated Poisson method but quoting the aberration yield in the irradiated fraction and the fraction of irradiated cells that was seen (columns 4 and 5). For comparison the true doses and fractions were used together with an assumed D_o of 2.5 Gy to calculate the expected fraction irradiated and seen and this is shown in column 3. Comparison of the values in columns 3 and 5 shows a reasonable agreement between expected and the observed fraction of irradiated cells that were seen at metaphase. The derived yield of dicentrics plus rings to the irradiated fraction (column 4) should be independent of the size of the irradiated fraction and thus the values should not change as one goes down the column. Weighted means of the yields at each dose are shown and also those points that differ from the mean by more than two standard errors are indicated.

In Table 8 the results of an analysis for the data for total aberrations is shown. Because it is known that total aberrations are overdispersed compared with Poisson (Edwards et al 1979) and the four distributions of total

aberrations for 100% irradiated samples shown in Tables 1 and 2 are all overdispersed (σ^2/y = 1.23, 1.32, 1.24 and 1.48), the assumption that the Poisson distribution applies to total aberrations in cells which have been irradiated breaks down. As shown in the Appendix this deficiency may be overcome by describing the overdispersion by the negative binomial. This may be specified using two parameters, the mean Y and a dispersion parameter, θ. The parameter $1/\theta$ is the ratio of variance to mean, so that θ must lie between zero and 1. When analysing the data allowing all the parameters, Y, θ and F (fraction of cells irradiated) to float, it was not always possible to obtain a sensible solution. By fixing the value of θ it was possible to obtain sensible solutions in most instances. The value chosen for θ was 0.9 based on our previous experience of measured values for the ratio of variance to mean for total aberrations produced by X and gamma rays (Edwards et al 1979). This corresponds to a ratio of variance to mean for 100% irradiated samples of 1.11. In practice, the derived yields and fractions irradiated were not very dependent upon the choice of θ. On three occasions (5 Gy Chilton, 100% irradiated and 8 Gy both laboratories 100% irradiated), it was not possible to arrive at a solution. On three other occasions (Chilton 8 Gy 97%, 90% and 70%) the numerical minimisation failed (see Appendix) but sensible solutions were obtained using θ = 0.89. The reasons for these problems are not yet understood, but they are related to the way in which the NAG library routine is being used. The other problem of accounting for a finite yield at zero dose was solved by using a background yield of 0.0035 aberrations per cell (see Appendix for details). If the yield equation for total aberrations (eq. 2 above) is used then doses of 5 and 8 Gy lead to expected yields of 3.0 and 7.5 aberrations per cell respectively, which are in reasonable agreement with the yields from Chilton given in Table 8 (column 4). The corresponding values from Leiden (column 6) are lower just as they were for dicentrics. Nevertheless, the consistency of the yields to the irradiated fractions in Table 8 and the

reasonable consistency between the expected (column 3) and estimated (columns 5 and 7) fractions of cells irradiated and seen at metaphase leads to the conclusion that total unstable aberrations as well as dicentrics and rings, can also be used to predict the size of the irradiated fraction and its dose.

It is interesting to observe from Table 1 that some cells have no aberrations in them, even for 100% irradiation at 5 Gy (11 in 171 from Chilton, 25 in 300 from Leiden). At 8 Gy however (Table 2) then in the 100% irradiated samples no cell free of aberrations was seen but when only 1% of cells were spared, then 6 in 33 (Chilton) and 22 in 100 (Leiden) were observed free from aberrations. This raises the possibility that if some lymphocytes are seen free from aberrations it is likely that in vivo some stem cells could remain to multiply and cause a host versus graft interaction after a bone marrow transplant. It would appear that the number of cells seen without aberrations could be used as a simple indicator of the likelihood of some cells having been spared from lethal exposure.

Micronuclei

Interpretation of the micronucleus data is rather more difficult than for chromosomal aberrations. There has been insufficient experience with the technique to measure reliable dose response curves and indeed Bauchinger (1990) has reviewed published data and showed considerable disparity. A quantitative estimate of how overdispersion varies with dose is not available. The only information on this is given by Prosser et al (1988) and Littlefield et al (1989) and the results are variable. The analysis presented here will therefore be to find parameters of the contaminated negative binomial distribution and to compare with estimates derived from the known mixtures. The control yield of micronuclei is taken as 0.012 which is the average of the zero dose observations in Tables 3 and 4. For the 100% irradiated specimens the ratios of variance to mean (Tables 3 and 4, Chilton and Leiden) were respectively 1.37 $\pm$ .13, 1.16 $\pm$ .04, 1.04 $\pm$ .08, 1.25 $\pm$ .04. These average out to about 1.2 for

the four measurements. It was therefore decided to fix the parameter θ at 0.8 for the purpose of the analysis of the results which is shown in Table 9.

For both laboratories dose effect curves for micronuclei exist (Prosser et al (1988) for Chilton and the Leiden curve is unpublished).

For Chilton $Y = .0129 + .117D + .0087D^2$ ...(3)

For Leiden $Y = .024 + .157D + .031D^2$ ...(4)

Table 9 shows for each dose and irradiated fraction the derived yields in columns 4 and 7, the derived doses in columns 5 and 8 and the fractions irradiated and seen in columns 6 and 9. The yields in columns 4 and 7 should depend only on the dose to the irradiated fraction. Weighted means have been calculated and chi-squared values are considerably greater than the corresponding statistics for chromosomal aberrations in Tables 6, 7 and 8. From the above dose effect curves, the dose is poorly predicted. The only set that predicts the dose within reason is for Chilton at 8 Gy, although this could be spurious because the curve has been extrapolated beyond the range of the data from which it was derived. The Chilton laboratory seriously overestimates the 5 Gy dose and Leiden seriously underestimates both doses. The deduced fractions of cells irradiated and seen by Chilton (column 6) are in good agreement with the expected values in column 3. The fractions deduced by Leiden however, are higher than expected and at 5 Gy show no smooth downward trend. Overall the micronucleus technique does not give such reliable results as the aberration endpoint. It is clear that, at present, consistency in deriving curves is lacking probably because the technique is relatively new. Details of cell culture still need to be standardised both within and between laboratories. Comparison of eqns 3 and 4 above indicate that Leiden obtains a higher dose response yet in the present work at both doses and all mixtures Chilton reports higher micronucleus yields. It is notable that by contrast to unstable aberrations even at 8 Gy 100% irradiated (Table 4) both laboratories observe a considerable number of binucleate cells free from micronuclei.

Prematurely condensed chromosomes

The PCC technique was carried out only at Leiden where the group has investigated the dose response relationship (unpublished). With fusion one day after irradiation a relationship over the range 1-8 Gy was obtained that fitted best to a weighted linear regression $Y = (1.45 \pm 0.03)D$. The results of the PCC experiments using samples irradiated at Chilton are shown in Tables 5a, b. Here it may be seen that for zero dose control values each cell scored contained 46 chromosomal pieces corresponding to the human diploid number. At both 5 and 8 Gy 100% irradiated the number of cells containing 46 chromosomal pieces was zero. At these doses therefore, there is good separation of irradiated and unirradiated cells. At 5.0 Gy (Table 5a) the yield in the irradiated fraction (final column) is constant for each sample mixture independant of the fraction irradiated. The corresponding values at 8.0 Gy (Table 5b) are also quite similar although there may be a slight tendency for the values to rise with increasing fraction irradiated. The averages of the values in the final columns are 6.26 and 11.35 excess PCC fragments per cell which from the linear regression above lead to dose estimates of 4.3 and 7.8 Gy. At both doses the percentages of cells damaged (penultimate column) correspond very closely to the percentages of cells irradiated (column 1). This indicates that with the PCC technique, there is no effect corresponding to the interphase death that operates with chromosomal aberrations and micronuclei. It follows therefore that in the situation of a small spared fraction one would expect to see more undamaged cells by the aberration or micronucleus assays and indeed inspection of the 95-99% irradiated values in Tables 2 and 4 shows this to be so. Nevertheless Tables 5a and b show that in the 95-97% irradiated material PCC cells without fragments were observed. It also follows that in the opposite situation of a localised burn the PCC method could be a more effective assay than aberrations or micronuclei.

Conclusions

This work has been done by in vitro mixing of irradiated and unirradiated cells to simulate a partial body exposure. It is therefore a much more simplified situation than that which applies in vivo where the distribution of doses over the body would probably form a continuum rather than two homogeneous fractions. The simplification also has taken no account of the distribution and circulation characteristics of lymphocytes or any other physiological processes that may operate on lymphocyte populations in vivo. Nevertheless, the work has demonstrated that it should be possible to use biological dosimetry to give a more refined indication of a high, but non-uniform irradiation, than simply quoting an estimate of averaged equivalent whole body dose. Potentially the methods described here may be able to provide the physician with sufficient information to aid in taking decisions regarding the management of patients thought to be at risk of severe depletion or destruction of their bone marrow. It is clearly necessary to follow up the work described here by in vivo studies of partially or inhomogeneously irradiated persons.

We have shown that by analysing the distribution data using the contaminated Poisson or Qdr methods for dicentrics or the contaminated negative binomial for total aberrations, reasonable estimates of the size of an irradiated fraction of lymphocytes and its dose, can be deduced. A comparison of the deduced values with the known values shows no particular advantage whether the calculations have been done on data for observed dicentric aberrations alone, or all unstable aberrations combined. On balance however, it is easier to handle the data on dicentrics because one may assume an underlying Poisson distribution of the aberrations. The opportunity has been taken in this paper to present in an Appendix in detail the algebraic steps that are involved in the calculations of the contaminated Poisson method. This technique has been outlined before (eg. IAEA 1986) but never fully described. A contaminated negative binomial method has been developed here and is

presented for the first time to enable similar calculations for endpoints such as total unstable aberrations or micronuclei that have a natural underlying tendency for overdispersed distributions even with homogeneous irradiation.

A disturbing feature of the results in the present work is the difference in aberration yields reported by the two laboratories that shared aliquots of the same samples of blood. This points to the need, often cited previously, (eg. IAEA, 1986) that interpretation of aberration yields in terms of dose should be done using in vitro curves constructed in the same laboratory. The lack of appropriate curves from Leiden has thus limited full derivation of values in the present work. Nevertheless, presentation of the data from that laboratory in terms of observed aberration yields compared with expected in the irradiated fraction and the calculation of the size of the fraction has shown that as for Chilton it is possible to distinguish even a few percent of spared cells mixed with cells that have been highly irradiated. This esssentially arises because of the selective advantage that unirradiated cells have in preferentially reaching metaphase by 48 hours in culture.

It should be noted that even for 95% of cells irradiated at 5 Gy there was still about 40% of cells seen that did not contain any aberrations and so were apparently undamaged. At 8 Gy and 99% irradiated about 20% of cells seen were normal. By contrast for 100% irradiated, at 5 Gy about 8% and at 8 Gy no cells were seen to be free from aberrations. This information in itself is of immediate qualitative value as an indication that some proportion of the cells has been spared. The implication from this is that there is likely to be natural recovery of bone marrow. For the analysis of a highly irradiated subject this would be apparent after relatively few cells had been scored. The clinician could then be informed early on with the expectation that when a larger sample has been analysed a more quantitative estimate of inhomogeneous irradiation would be available using the methods described in this paper.

The analysis of the results from the micronucleus measurements of both laboratories indicate that this endpoint is less reliable than aberrations.

The data showed even more variation both inter and intra-laboratory and less concordance with the expected values deduced from the known doses and sizes of irradiated fractions. Far less research has gone into characterising the dose response for the newer micronucleus method compared with dicentrics and this is reflected in the currently rather disparate published data. At present we see no significant advantage in using the micronucleus assay as a biological dosemeter for the particular purpose addressed by this paper of determining inhomogeneous exposure.

Results for the prematurely condensed chromosome assay suggest that there is no selective advantage for a small fraction of spared cells being preferentially scored. This is because the phenomena of interphase death and mitotic delay do not appear to operate with this assay. For detecting partial body irradiation within two days of an accident the PCC technique seems to be efficient. The number of normal cells observed reflects more accurately the proportion of unirradiated lymphocytes in the mixture without the need for correction. PCC may be the method of choice for the localised burn.

Acknowledgement

We are indebted to David Papworth of the UK Medical Research Council's Radiobiology Unit, Harwell who devised the method of deriving the fraction irradiated and its mean from a contaminated Poisson distribution. Although this procedure has been available for several years it has never formally been published. We are taking the opportunity here to present the algebra in full. Those ideas have also been applied here, for the first time, to the contaminated negative binomial distribution. We are grateful to David Papworth for critically reviewing this appendix.

Table 1

Results for aberrations in cells irradiated at 5 Gy

% of cells irradiated at 5 Gy	Cells scored	Dicentrics	Centric rings	Acentrics	Dicentric distribution								Total aberration distribution										
					0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	8	9	10
0	500	0	0	1	500								499	1									
30	451	32	6	28	430	11	9	1					424	10	5	5	6	0	0	1			
50	247	45	3	32	225	4	13	5					216	10	7	4	6	4					
70	200	81	5	59	162	15	12	6	3	0	2		158	7	9	11	5	3	3	1	1	1	1
80	200	102	13	67	154	17	12	11	3	2	1		144	13	11	6	11	6	8	1			
90	120	103	9	38	80	12	7	13	4	3	0	1	73	9	10	11	8	3	2	3	1		
95	257	239	3	180	131	57	40	20	4	4	1		103	44	30	29	30	16	4	1			
100	171	282	14	208	36	53	46	16	15	1	4		11	26	42	40	22	11	8	6	4	1	

(a) Chilton

0	200	0		6	200								194	6									
30	200	6		10	195	4	1						188	8	4								
50	200	36		76	182	7	4	7					154	24	6	5	2	3	4	2			
70	200	47		71	166	21	13						144	21	17	10	7	1					
80	200	58		101	157	30	11	2					132	19	26	10	9	3	0	1			
90	200	96		140	131	46	19	4					107	24	33	10	18	5	2	1			
95	100	50		100	65	22	11	2					50	12	14	8	6	2	4	4			
100	300	312		454	112	92	69	26	1				25	71	88	33	37	19	16	7	4		

(b) Holden

Table 2

Results for aberrations in cells irradiated at 8 Gy

% of cells irradiated at 8 Gy	Cells scored	Dicentrics	Centric rings	Acentrics	Dicentric distribution													Total aberration distribution															
					0	1	2	3	4	5	6	7	8	9	10	11	12	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
0	100	0	0	0	100													100															
30	200	16	2	8	196	0	0	0	4									196	0	0	0	1	0	0	2	1							
50	300	35	0	23	293	0	0	1	3	1	0	1	1					292	0	1	1	0	1	0	1	0	1	2	0	1			
70	438	129	7	101	402	3	8	12	3	5	1	1	3					398	2	2	5	2	8	6	5	4	0	2	3	1			
80	400	181	6	113	356	3	6	7	13	5	6	2	1	0	1			352	5	1	4	7	2	7	3	8	4	4	0	2	0	0	0	1
90	300	227	16	132	239	5	13	11	14	8	4	5	1					236	0	3	6	11	8	10	13	7	2	2	2					
95	146	253	12	145	83	5	9	16	11	9	8	1	1	2	0	0	1	80	1	4	5	6	11	13	7	8	3	4	1	2	0	1		
97	50	105	3	45	27	0	2	4	7	3	4	2	1					26	0	0	1	1	5	8	5	2	1	0	0	0	1			
99	33	102	4	47	6	1	8	5	3	5	3	1	1					6	0	2	3	5	4	3	4	3	0	3						
100	21	100	4	45	0	0	3	2	4	6	2	3	0	1				0	0	2	1	1	2	2	3	5	1	0	2	2				

(a) Chilton

0	100	0		3	100	0												97	3														
30	100	0		4	100	0												96	4														
50	100	7		22	96	1	3											91	3	0	3	1	1	0	0	1							
70	100	42		54	87	1	3	4	2	3								75	6	4	3	3	3	4	2	1							
80	100	52		66	82	3	5	5	2	2	1							70	6	4	4	4	4	3	3	1	1						
90	100	76		82	73	8	4	6	4	4	1							59	7	10	5	4	4	4	4	1	1	0	1				
95	100	110		154	58	7	16	10	4	5								46	8	6	4	4	9	8	7	3	2	1	2				
97	100	98		174	49	19	21	8	2	1								40	9	6	6	10	6	9	7	4	2	1					
99	100	136		226	35	20	25	15	4	1								22	8	10	9	12	12	12	4	6	2	1	1	1			
100	150	292		440	17	38	54	25	11	3	2							0	15	21	16	19	20	19	11	15	6	4	2	2			

(b) Ixidon

Table 3

Results for micronuclei in binucleate cells at 5 Gy

% of cells irradiated	Cells scored	MN	Distribution 0	1	2	3	4	5	6	7	8
0	500	3	498	1	1						
30	500	64	464	20	8	4	4				
50	500	101	445	31	11	7	4	1	1		
70	1000	302	825	86	64	15	7	3			
80	1000	394	778	108	72	29	11	1	1		
90	500	185	379	76	29	13	3				
95	500	818	146	101	109	96	31	15	2		
100	300	506	79	73	68	48	17	8	5	1	1
			(a) Chilton								
0	1000	14	986	14							
30	1000	110	937	30	20	12	1				
50	1000	198	879	70	28	20	3				
70	1000	278	782	166	44	8					
80	1000	426	706	188	80	26					
90	1000	456	666	230	86	18					
95	1000	542	636	220	112	30	2				
100	1000	705	523	308	123	35	10	0	1		
			(b) Leiden								

Table 4

Results for micronuclei in binucleate cells at 8 Gy.

% of cells irradiated	Cells scored	MN	Distribution 0	1	2	3	4	5	6	7	8
0	500	6	494	6							
50	500	26	488	6	3	0	2	0	1		
70	500	57	477	9	6	1	3	3	1		
80	500	118	445	18	16	16	5				
90	500	188	408	27	42	17	4	2			
95	500	344	328	71	49	39	9	2	2		
97	400	347	225	73	57	29	7	9			
99	283	344	118	56	63	29	13	2	1	1	
100	300	517	63	81	71	56	20	8	1		
(a) Chilton											
0	500	7	493	7							
50	500	105	430	45	15	10					
70	500	170	400	55	25	15	5				
80	500	250	370	65	30	20	10	5			
90	500	287	331	90	40	39					
95	400	264	248	84	36	20	12				
97	1000	746	562	236	132	44	18	6	2		
99	1000	826	496	302	126	48	18	4	6		
100	1000	1184	332	360	184	70	32	14	8		
(b) Leiden											

Table 5a

Results for premature chromosome condensation at 5 Gy

% of cells irradiated	PCC distribution																									Excess PCC	Cells scored	Cells damaged	% cell damage	mean per damaged cell
	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69						
0	95																									0	95	0	0	
10	96	0	1	0	2	3	3	2	1	1	1															84	110	14	11.3 ± 3.2	6.00 ± .65
30	73	0	0	1	7	6	4	3	0	1	2	1														146	98	25	20.3 ± 4.4	5.84 ± .48
50	24	0	0	1	4	5	3	2	1	1	1	0	1													115	43	19	44.2 ± 7.6	6.05 ± .56
70	13	0	0	0	9	12	8	7	2	1	3															248	55	42	76.4 ± 5.7	5.90 ± .37
80	9	0	0	0	8	9	10	9	3	1	1	1	1													266	52	43	82.7 ± 5.2	6.19 ± .38
90	6	0	0	0	7	12	12	13	4	1	2	2	1													346	60	54	90.0 ± 3.9	6.41 ± .34
95	3	0	0	0	10	12	9	7	7	3	1	1	2													331	55	52	94.5 ± 3.1	6.37 ± .35
97	2	0	0	0	7	8	11	8	6	3	0	1	1	1	1											315	49	47	95.9 ± 2.8	6.70 ± .38
99	0	0	0	0	6	9	10	10	6	4	1	2	1	1												340	50	50	100	6.80 ± .37
100	0	0	0	1	27	33	30	20	11	7	3	3	3	1	2											887	141	141	100	6.29 ± .21

Table 5b

Results for premature chromosome condensation at 8 Gy

% of cells irradiated	PCC distribution																								Excess PCC	Cells scored	Cells damaged	% cell damage	mean per damaged cell
	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69					
0	156																								0	156	0	0	
10	87	0	0	0	0	1	1	2	1	1	2	1	1	1	0	1	0	0	0	0	0	1			134	100	13	13.0 ± 3.4	10.3 ± .9
30	65	0	0	0	0	0	0	0	2	4	4	4	2	0	3	1	2	0	2	1	1				324	91	26	28.6 ± 4.7	12.5 ± .7
50	28	0	0	0	0	0	1	2	5	3	2	0	0	1	0	1	0	0	0	0	1	1	1		198	46	18	39.1 ± 7.2	11.0 ± .8
70	14	0	0	0	0	0	0	5	7	9	7	4	3	1	2	1	2								410	55	41	74.5 ± 5.9	10.0 ± .5
80	10	0	0	0	0	0	2	9	6	3	4	9	4	3	1	2	3								468	56	46	82.1 ± 5.1	10.2 ± .5
90	6	0	0	0	0	0	1	9	8	4	3	9	4	2	4	1	4	1	1						542	57	51	89.5 ± 4.1	10.6 ± .5
95	3	0	0	0	0	0	1	5	8	3	1	5	3	3	2	4	3	1	2	2	2				539	48	45	93.8 ± 3.5	12.0 ± .5
97	1	0	0	0	0	0	0	5	5	5	4	4	3	4	7	5	2	3	2	0	0	1	1		627	52	51	98.1 ± 1.9	12.3 ± .5
99	0	0	0	0	0	0	0	1	7	4	7	4	4	5	3	1	2	2	1	1	0	2	1		550	45	45	100	12.2 ± .5
100	0	0	0	0	0	0	0	5	10	9	13	7	5	5	2	3	4	3	4	1	2	2	2	2	979	79	79	100	12.4 ± .4

Table 6

Analysis of results for dicentrics from Chilton

Dose	True fraction irradiated	Expected fraction irradiated and seen D_o = 2.5 Gy	Contaminated Poisson			Qd		
			Estimated dose	Estimated fraction irradiated and seen	Estimated fraction irradiated	Estimated dose	Estimated fraction irradiated and seen	Estimated fraction irradiated
5 Gy	1.00	1.00	4.80 ± .19	.96 ± .05	.99 ± .01	4.70	1.00	1.00
	0.95	0.72	4.40 ± .20+	.64 ± .05	.912 ± .014+	4.31	0.67	0.92
	0.9	0.55	5.63 ± .34	.37 ± .05	.85 ± .03	5.38	0.40	0.85
	0.8	0.35	5.04 ± .32	.27 ± .04	.74 ± .04	4.80	0.30	0.74
	0.7	0.24	4.88 ± .36	.23 ± .04	.68 ± .05	4.98	0.22	0.68
	0.5	0.12	4.71 ± .48	.11 ± .02	.45 ± .06	4.10	0.14	0.46
		Weighted mean	4.80					
		X^2	10.6					
		DF	5					
8 Gy	1.00	1.00	*8.18 ± .40	1	1	8.18	1.00	1.00
	0.99	.80	7.16 ± .39+	.84 ± .07	.99 ± .01	7.25	.82	.99
	0.97	.57	7.96 ± .41	.47 ± .07	.96 ± .01	7.83	.48	.96
	0.95	.44	7.42 ± .25+	.44 ± .04	.94 ± .01	7.31	.45	.94
	0.90	.27	7.10 ± .26+	.21 ± .02	.82 ± .03+	7.01	.21	.82
	0.80	.140	7.52 ± .30	.112 ± .016	.72 ± .04	7.24	.12	.71
	0.70	.087	6.95 ± .34+	.085 ± .014	.60 ± .05	6.67	.092	.59
	0.50	.039	8.36 ± .74	.023 ± .009	.41 ± .12	7.83	.027	.39
		Weighted mean	7.42					
		X^2	10.9					
		DF	7					

* This sample was underdispersed (σ^2/y = 0.31) and so the irradiated fraction is assumed to be 1.

+ Estimates that deviate from the true values by more than 2 standard errors.

The uncertainties shown are standard errors.

Table 7

Analysis of results for dicentrics and rings from Leiden

Dose	True fraction irradiated	Expected fraction irradiated and seen D_o = 2.5 Gy	Estimated yield to fraction irradiated	Estimated fraction irradiated and seen
8 Gy	1.0	1.0	*1.95 ± .11	1
	.99	.80	1.72 ± .19	.79 ± .07
	.97	.57	1.49 ± .20+	.66 ± .08
	.95	.44	2.38 ± .26	.46 ± .06
	.90	.27	2.61 ± .34	.29 ± .05
	.80	.140	2.70 ± .42	.19 ± .04
	.70	.087	3.08 ± .52+	.14 ± .04
	.50	.039	1.25 ± .67	.06 ± .03
		Weighted mean	1.96	
		χ^2	22.3	
		DF	7	
5 Gy	1.0	1.0	*1.04 ± .06	1
	.95	.72	.76 ± .19	.66 ± .14
	.90	.55	.70 ± .13	.68 ± .11
	.80	.35	.63 ± .16	.46 ± .10
	.70	.24	.69 ± .18	.34 ± .08
	.50	.12	1.59 ± .35	.11 ± .03
		Weighted mean	0.93	
		χ^2	16.2	
		DF	5	

*Distributions were underdispersed and so fraction irradiated was assumed to be 1.

+Estimates that deviate from the true values by more than 2 standard errors.

The uncertainties shown are standard errors.

Table 8

Analysis of results for total unstable aberrations

Dose	True fraction irradiated	Expected fraction irradiated and seen	Chilton		Leiden	
			Estimated yield to irradiated fraction	Estimated fraction irradiated and seen	Estimated yield to irradiated fraction	Estimated fraction irradiated and seen
5 Gy	1.0	1.0	2.95 ± .16	1.00 ± .02	2.55 ± .10	1
	.95	.72	2.49 ± .15	.66 ± .04	2.78 ± .27	.54 ± .06
	.9	.55	3.02 ± .29	.41 ± .05	2.24 ± .18	.53 ± .04
	.8	.35	3.10 ± .27	.29 ± .03	2.00 ± .21	.40 ± .04
	.7	.24	3.32 ± .32	.22 ± .03	1.70 ± .22	.35 ± .04
	.5	.12	2.34 ± .34	.14 ± .02	2.09 ± .26	.26 ± .04
		Weighted mean	2.80		2.34	
		X^2	11.4		19.4	
		DF	5		5	
8 Gy	1.0	1.0	7.10 ± .61	1	4.88 ± .19	1
	.99	.80	5.65 ± .49	.82 ± .07	4.57 ± .26	.79 ± .04
	.97	.57	6.39 ± .55	.48 ± .07	4.48 ± .30	.60 ± .05
	.95	.44	6.21 ± .33	.45 ± .04	4.86 ± .33	.54 ± .05
	.90	.27	5.85 ± .32	.21 ± .02	3.74 ± .34	.42 ± .05
	.80	.140	6.65 ± .44	.11 ± .02	3.87 ± .41	.30 ± .05
	.70	.087	6.07 ± .43	.089 ± .014	3.73 ± .44	.25 ± .05
	.50	.039	7.22 ± 1.01	.027 ± .009	3.21 ± .75	.088 ± .031
		Weighted mean	6.20		4.48	
		X^2	6.9		18.6	
		DF	7		7	

Table 9

Analysis of results for micronuclei

Dose	True fraction irradiated	Expected fraction irradiated and seen	Chilton			Leiden		
			Estimated yield to irradiated fraction	Estimated dose	Estimated fraction irradiated and seen	Estimated yield to irradiated fraction	Estimated dose	Estimated fraction irradiated and seen
5 Gy	1.00	1.00	1.86 ± .12	9.4 ± .4	.91 ± .04	.71 ± .03	2.9 ± .1	1
	0.95	0.72	1.92 ± .10	9.6 ± .4	.86 ± .03	.70 ± .07	2.9 ± .2	.77 ± .07
	0.90	0.55	0.77 ± .13	4.8 ± .6	.47 ± .07	.48 ± .07	2.1 ± .2	.95 ± .12
	0.80	0.35	1.18 ± .11	6.7 ± .5	.33 ± .03	.64 ± .08	2.7 ± .2	.66 ± .07
	0.70	0.24	1.12 ± .12	6.5 ± .5	.26 ± .03	.32 ± .08	1.6 ± .3	.88 ± .23
	0.50	0.12	1.34 ± .25	7.4 ± 1.0	.14 ± .03	1.02 ± .15	3.7 ± .4	.19 ± .03
		Weighted mean	1.42			0.65		
		χ^2	75			34		
		DF	5			5		
8 Gy	1.00	1.00	1.72 ± .05	8.9 ± 0.2	1	1.18 ± .04	4.1 ± .1	1
	0.99	0.80	1.60 ± .14	8.4 ± 0.5	.76 ± .05	0.90 ± .07	3.4 ± .2	.91 ± .05
	0.97	0.57	1.46 ± .13	7.9 ± 0.5	.60 ± .04	1.03 ± .07	3.8 ± .2	.72 ± .04
	0.95	0.44	1.50 ± .13	8.0 ± 0.5	.46 ± .04	1.09 ± .13	3.9 ± .3	.60 ± .06
	0.90	0.27	1.62 ± .19	8.5 ± .07	.23 ± .03	1.05 ± .12	3.8 ± .3	.56 ± .05
	0.80	0.140	1.83 ± .26	9.3 ± 1.1	.127 ± .019	1.39 ± .15	4.6 ± .3	.35 ± .03
	0.70	0.087	2.67 ± .54	12.0 ± 1.7	.039 ± .010	1.07 ± .16	3.9 ± .4	.31 ± .04
	0.50	0.039	2.66 ± .86	12.0 ± 2.6	.016 ± .007	0.76 ± .18	3.0 ± .5	.27 ± .06
		Weighted mean	1.80			1.09		
		χ^2	21.3			20.7		
		DF	7			7		

APPENDIX

The derivation of the parameters of a contaminated Poisson distribution from an observed distribution of dicentrics

The problem

Suppose that the following distribution of dicentrics is observed among the cells. The distribution is tested for conformity with Poisson by a test such as Papworth's (1970) 'u' test and shown to be overdispersed. The variance therefore is significantly greater than the mean.

cells with dicentrics						
0	1	2	...	i	.....	r
n_0	n_1	n_2	...	n_i	.....	n_r

Let the total number of cells be n

$$n = \sum_{i=0}^{r} n_i$$

Let the total number of dicentrics be x

$$x = \sum_{i=0}^{r} i\, n_i$$

The hypothesis is that the distribution can be described by a mixture of a fraction p of a Poisson distribution of mean m and a fraction (1-p) of zeros. It is assumed that unirradiated cells contain no dicentrics.

The expected distribution is:

$$P(0) = 1-p + pe^{-m}$$

$$P(i) = \frac{pm^i e^{-m}}{i!} \qquad \text{for } i \geq 1$$

One would wish to find estimates for p and m which best fit the observed distribution.

The maximum likelihood solution

The best estimates of p and m are assumed to be those which maximise the likelihood L of the observations. In practice it is easier to solve the equations which maximise the log-likelihood. Formally the maximum occurs when equations (1) and (2) are satisfied

$$\frac{\partial \log L}{\partial p} = 0 \qquad (1)$$

and

$$\frac{\partial \log L}{\partial m} = 0 \qquad (2)$$

Furthermore, asymptotic estimates of the variance on m, var(m), the variance on p, var(p), and the covariance of m and p, covar(m,p), can be obtained using equation (3)

$$\begin{bmatrix} \text{var}(p) & \text{covar}(m,p) \\ \text{covar}(m,p) & \text{var}(m) \end{bmatrix} = \begin{bmatrix} -\frac{\partial^2 \log L}{\partial p^2} & -\frac{\partial^2 \log L}{\partial p \partial m} \\ -\frac{\partial^2 \log L}{\partial p \partial m} & -\frac{\partial^2 \log L}{\partial m^2} \end{bmatrix}^{-1} \qquad (3)$$

In this case it is possible to solve these equations analytically and this is laid out below.

The likelihood L of the observations is

$$L = \prod_{i=0}^{r} P(i)^{n_i}$$

$$= (1-p+pe^{-m})^{n_o} \prod_{i=1}^{r} \left(\frac{pm^i e^{-m}}{i!}\right)^{n_i}$$

$$= (1-p+pe^{-m})^{n_o} \, p^{n-n_o} m^x e^{-(n-n_o)m} \Big/ \prod_{i=1}^{r} (i!)^{n_i}$$

$$\therefore \log L = n_o \log(1-p+pe^{-m}) + (n-n_o)\log p + x \log m - (n-n_o)m - \sum_{i=1}^{r} n_i \log i!$$

$$\frac{\partial \log L}{\partial p} = \frac{n_o(e^{-m}-1)}{1-p+pe^{-m}} + \frac{n-n_o}{p} \qquad (4)$$

$$\frac{\partial \log L}{\partial m} = \frac{n_o(-pe^{-m})}{1-p+pe^{-m}} + \frac{x}{m} - (n-n_o) \qquad (5)$$

At the position of maximum likelihood expressions (4) and (5) are zero

$$\therefore \quad \frac{n_o(e^{-m}-1)}{1-p+pe^{-m}} + \frac{n-n_o}{p} = 0 \qquad (6)$$

$$\frac{n_o(-pe^{-m})}{1-p+pe^{-m}} + \frac{x}{m} - (n-n_o) = 0 \qquad (7)$$

Divide (7) by p and add to (6)

$$1 - p + pe^{-m} = \frac{mpn_o}{x} \qquad (8)$$

Substitute in equation (6)

$$\frac{m}{1-e^{-m}} = \frac{x}{n-n_o} \qquad (9)$$

From (6)

$$\frac{1 - p(1-e^{-m})}{n_o(1-e^{-m})} = \frac{p}{n-n_o}$$

$$\therefore \quad \frac{1}{n_o(1-e^{-m})} = \frac{p}{n-n_o} + \frac{p}{n_o} = \frac{pn}{n_o(n-n_o)}$$

Substitute for $1-e^{-m}$ from (9)

$$\frac{x}{n_o(n-n_o)m} = \frac{pn}{n_o(n-n_o)}$$

$$\therefore \quad mp = \frac{x}{n} \qquad (10)$$

Equations (9) and (10) can be solved to produce maximum likelihood estimates $\hat{m}$ and $\hat{p}$. As far as we can determine these equations first appeared in Pershad et al (1961) but without a derivation.

To estimate variances on $\hat{m}$ and $\hat{p}$, second derivatives are required. From (4) and (5)

$$\frac{\partial^2 \log L}{\partial p^2} = -\frac{n_o(e^{-m}-1)^2}{(1-p+pe^{-m})^2} - \frac{(n-n_o)}{p^2}$$

$$\frac{\partial^2 \log L}{\partial p \partial m} = \frac{(1-p+pe^{-m})(-n_o e^{-m}) - n_o(e^{-m}-1)(-pe^{-m})}{(1-p+pe^{-m})^2}$$

$$= -\frac{n_o e^{-m}}{(1-p+pe^{-m})^2}$$

$$\frac{\partial^2 \log L}{\partial m^2} = - \frac{n_o p[(1-p+pe^{-m})(-e^{-m}) - e^{-m}(-pe^{-m})]}{(1-p+pe^{-m})^2} - \frac{x}{m^2}$$

$$= \frac{n_o p(1-p)e^{-m}}{(1-p+pe^{-m})^2} - \frac{x}{m^2}$$

The variance on $\hat{m}$ and $\hat{p}$ can be evaluated by substituting these expressions into equation (3) using $\hat{m}$ and $\hat{p}$ for m and p respectively.

Having derived estimates of the yield $\hat{m}$ and the fraction of cells which were irradiated and observed $\hat{p}$ it is necessary to calculate the dose D to the irradiated fraction and the fraction of cells irradiated taking into account interphase death, mitotic delay and any other process that selectively prevents an irradiated cell being observed at metaphase in 48h. For brevity, these will in future be called interphase death.

The yield m is related to dose D by equation (11) and so $\hat{D}$ can be derived

$$m = C_1 + \alpha D + \beta D^2 \qquad (11)$$

The variance of $\hat{D}$, var($\hat{D}$) is given by differentiating equation (11)

$$\text{var}(\hat{D}) = \frac{\text{var}(\hat{m})}{(\alpha + 2\beta\hat{D})^2} \qquad (12)$$

The fraction irradiated F is related to the dose D and the fraction p by equation (13) where D_o is the dose required to reduce the number of irradiated cells by a factor e due to interphase death.

$$F = \frac{pe^{D/D_o}}{1-p+pe^{D/D_o}} = \frac{e^{D/D_o}}{e^{D/D_o} - 1+\frac{1}{p}} \qquad (13)$$

The variance on $\hat{F}$, var($\hat{F}$) is given by differentiating (13)

$$\text{var}(\hat{F}) = \left(\frac{\partial F}{\partial p}\right)^2 \text{var}(\hat{p}) + \left(\frac{\partial F}{\partial D}\right)^2 \text{var}(\hat{D}) + 2 \frac{\partial F}{\partial p} \frac{\partial F}{\partial D} \text{covar}(\hat{p},\hat{D}) \qquad (14)$$

In equation (14)

$$\frac{\partial F}{\partial p} = - \frac{e^{D/D_o}}{p^2(\frac{1}{p} - 1 + e^{D/D_o})^2}$$

$$\frac{\partial F}{\partial D} = \frac{(1-p)e^{D/D_o}}{pD_o\ (\frac{1}{p} - 1 + e^{D/D_o})^2}$$

$$\text{cov}\ (\hat{p},\hat{D}) = \frac{\text{covar}(\hat{p},\hat{m})}{\alpha + 2\beta D}$$

This technique applies very well to distributions of dicentrics. However if the distribution of total aberrations or micronuclei are used two problems arise. Firstly the unirradiated fraction has a level of aberrations which typically may be about 1/300 for total aberrations and 1 or 2 in 100 for micronuclei. These may not be negligible whereas for dicentrics a typical background value is 1/1000 and this can be ignored and a yield of zero assumed for the unirradiated fraction. Secondly the distribution for the irradiated fraction is already overdispersed compared with Poisson. These can be taken into account by introducing a non zero control and assuming that the aberrations in the irradiated fraction follow a negative binomial distribution.

The negative binomial distribution

Two parameters are required s and θ

The probability that i aberrations occur in one cell is given by eq. 15

$$P(i) = \frac{(s + i - 1)!}{i!(s-1)!}\ \theta^s(1-\theta)^i \qquad ...(15)$$

The mean of this distribution m and its variance σ^2 are as follows

$$m = \frac{s(1-\theta)}{\theta}$$

$$\sigma^2 = \frac{s(1-\theta)}{\theta^2}$$

The parameter θ lies between 0 and 1 and will typically lie between 0.7 and 0.9 for total aberrations and micronuclei. The parameter s ought to be an integer but could be treated as a variable if the factorials are replaced by the appropriate Γ function.

At zero dose, the yield is so low that the chance of seeing a cell with two aberrations is negligible. The frequency distribution is given below where C is the control yield.

$$P(0) = 1 - C$$

$$P(1) = C$$

$$P(i) = 0 \quad i \geq 2$$

Thus the frequency distribution of a negative binomial (parameters s and θ) contaminated with a fraction (1-p) of the control yield C is shown in equations (16)

$$\left.\begin{aligned} P(0) &= (1-p)(1-C) + p\theta^s \\ P(1) &= (1-p)C + ps\theta^s(1-\theta) \\ P(i) &= \frac{p(s+i-1)!}{i!(s-1)!}\ \theta^s(1-\theta)^i \end{aligned}\right\} \qquad \ldots(16)$$

The log-likelihood logL of the observations n_o, n_1, n_2 etc is

$$\log L = \sum_{i=o}^{r} n_i \log(P(i))$$

The maximum likelihood equations are:-

$$\frac{\partial \log L}{\partial p} = 0$$

$$\frac{\partial \log L}{\partial \theta} = 0$$

$$\frac{\partial \log L}{\partial s} = 0$$

Solving these analytically is too complicated. They are therefore solved numerically. Second derivatives are also calculated numerically to use an equation like (4) to estimate variances. It is easier to interpret the output if the mean yield m of the negative binomial is used instead of the parameter s. This is done by replacing s by $\frac{m\ \theta}{1-\theta}$. Thus the variables are m, θ and p and may be determined using a minimising routine such as the NAG routine E04JBF. In practice - logL is minimised. Having obtained the yield m and the fraction p then the same procedure to deduce the dose D and the fraction F irradiated as described previously can be used again.

INSTITUUT VOOR TOEGEPASTE RADIOBIOLOGIE EN

IMMUNOLOGIE

HOOFDGROEP GEZONDHEIDSONDERZOEK TNO

CONTRACT BI6 - 0288 - NL

TREATMENT AND BIOLOGICAL DOSIMETRY OF EXPOSED PERSONS

D. N. VAN BEKKUM, G. WAGEMAKER.

TREATMENT AND BIOLOGICAL DOSIMETRY OF EXPOSED PERSONS

INTRODUCTION

The experience with the treatment of heavily irradiated victims of Chernobyl has led to the identification of a number of problems, some of which can most probably be solved by limited investigations employing already existing technology, others requiring relatively new approaches with less certainty in outcome. At the time of the Chernobyl accident, it was thought that patients who received a whole body dose exceeding the LD 90 can only be rescued by bone marrow transplantation. At lower doses, e.g. and LD 50, 50% of the patients will recover spontaneously and the others will not. How to distinguish between the two categories? Obviously such a distinction is even more important with lower radiation exposures. We propose to develop a prognostic test by measuring the hematological response of heavily irradiated rhesus monkeys to a short term treatment with certain hemopoietic growth factors. These factors, called GM-CSF, and Interleukin 3 (IL-3), stimulate the proliferation of primitive multipotent stem cells in vitro and may be expected to act similarly in vivo. The first project aimed at 1/ establishment of the relationship between irradiation dose given and growth factor response, and 2/ acceleration of hemopoietic reconstitution by growth factor treatment. In the short duration of this project, the hemopoietic growth factor GM-CSF (granulocyte/macrophage colony-stimulating factor) was studied most extensively, while the growth factor that stimulates hemopoietic stem cells, interleukin-3 (IL-3), was cloned specifically for monkey studies.

Autologous BMT has many advantages over allogeneic BMT, but is only feasible when the victims' stem cells have been collected and cryopreserved in advance. The techniques for bone marrow stem cell preservation have been worked out and are widely used in the treatment of leukemia. In those cases the stem cells are collected by multiple bone marrow punctures under general anaesthesia. This is not a desirable practice to apply to personnel at risk involved in reactor

operations. The group of Fliedner, Ulm, has shown that stem cells can be collected from the peripheral blood, but in order to collect sufficient cell numbers, cell mobilizing agents have to be administered. So far, this procedure is not without side effects and there is a need for the development of non-toxic mobilizers. Such research can best be performed in dogs, firstly because the experience has largely been gained with that species and, secondly, because the dog, due to its size, is more suitable for large scale collection of peripheral blood cells. A contribution from the Rijswijk group could be to study the possibility of expanding stem cell populations in vitro by stimulation with GM-CSF or IL-3. If such expansion would yield a substantial increase of the number of stem cells, smaller samples could be collected from the peripheral blood obviating the need for mobilizing agents. If that technique is promising it could perhaps also be applied to fetal liver cells, which are a potential source of donor material, but at the moment cannot be applied because the yield of stem cells per fetus is inadequate to achieve the repopulation of an adult recipient.

The second project thus aimed at expanding stem cell populations in vitro by stimulation with hemopoietic growth factors to facilitate engraftment following transplantation. This would in principle allow for relatively small samples of stem cells to be grown in vitro to full-scale autologous (or perhaps: allogeneic) grafts. To enable easy handling of bone marrow cells and to improve reproducibility of procedures, we set out with the development of a general method to select hemopoietic stem cells by positive identification.

I. Evalution of the potential for autogenous hemopoietic recovery of patients thought to have received a life-threatening whole body irradiation by means of hemopoietic growth factors

In this project, we have been testing in vivo administration of the hemopoietic growth factor GM-CSF for radioprotective effects, prognostic properties to assess the residual number of hemopoietic cells following exposure to total body irradiation (TBI), and for its capacity to accelerate immunohemopoietic reconstitution after transplantation of low numbers or autologous BM cells in rhesus monkeys. The study is also directed at dose-finding and side-effects.

In principle, there are three separate potential mechanisms, by which a (combination of) hemopoietic growth factor(s) may provide radioprotection: 1/ administration of HGFs before exposure to increase the numbers of peripheral blood cells, which results in a later onset of dangerous pancytopenia after exposure; 2/ administration of HGFs that increase the number of stem cells before exposure, from which a more rapid recovery of pancytopenia may be expected; 3/ administration of HGFs immediately after exposure, which may also lead to a more rapid recovery of pancytopenia by accelaration of the production rate of blood cells (Figure 1). Although only the latter mechanism is relevant in case of accidental high dose exposure, the full evaluation of HGFs as radioprotective agents involves experiments directed at all three putative mechanisms. To date, the dose-finding studies of GM-CSF and its radioprotective properties and effects on reconstitution following autologous BMT are largely completed.

Studies on the radiation sensitivity of primate stem cells, based on a comparison of the autogenous regeneration rate after graded doses of TBI with graded numbers of transplanted autologous bone marrow cells after high dose TBI, led to a to a reappraisal of the D_o value characterizing the radiation sensitivity of primate stem cells. A D_o value of 0.9 Gy (confidence limits 0.8 - 1.1) for orthovolt X-rays is now assumed rather than 0.6 Gy as was earlier calculated. In principle, a residual number of bone marrow stem cells capable of autogenous regeneration can now be assumed up to doses of 10 Gy TBI. This hypothesis was prospectively tested using extensive supportive care (gastrointestinal decontamination, antibiotic treatment, fluid and electrolyte administration and deliberate blood transfusions). The results demonstrated that the LD 50 of about 5 Gy, hitherto reported, will be shifted to about 9.5 Gy by providing this extensive supportive care. Even at high doses of TBI, hemopoiesis will ultimately (6 - 8 weeks after TBI) regenerate. This observation raises serious doubts on the validity of bone marrow transplantation as a means to rescue victims of high doses of accidental exposure to ionizing radiation and provides a scientific basis for attempts to accelerate hemopoietic reconstitution by growth factor treatment.

To test the efficacy of the hemopoietic growth factor GM-CSF, rhesus monkeys were irradiated with graded doses (4-10 Gy) of TBI (6 MV

X-rays) and treated with GM-CSF or placebo (days 1-14). Another group was given 2 x 6 Gy TBI (24 h interval), transplanted with a very small number of purified autologous HSC (equivalent to 10^7/kg unfractionated BM) and graded doses (3-100 ug/kg/day) of GM-CSF (days 1-30). All animals were subjected to complete supportive care. A shortening of 5 days on an average pancytopenic period of 3 weeks resulted from the GM-CSF treatment in the autologous BMT setting. Continuation of GM-CSF administration after regeneration resulted in a dose-dependent leukocytosis; 10 - 30 ug/kg/day GM-CSF appeared to be optimal, toxic side-effects were not observed. Since these results were rather disappointing, it was postulated that the response of the growth factor was directly related to the number of stem cells available for regeneration, which purposely was kept limited in this set of experiments. This was approached by titrating TBI dose without BMT. Following TBI without BMT, the efficacy of 30 ug/kg/day GM-CSF decreased sharply from 5 Gy TBI (complete prevention of 2 weeks of leukopenia) to 8 Gy (no significant clinical gain). Further evaluation was done by relating cumulated blood cell counts (i.e. the sum of blood cell counts over the period of observation to eliminate day-to-day variations and to get a fair relative account of total number of cells produced) for GM-CSF treated monkeys and placebo controls to the dose of radiation given. From this evaluation, it was clear, that white cell counts (mainly neutrophilic and eosinophilic granulocytes) were most effectively stimulated by GM-CSF, while reticulocytes hardly showed a response and thrombocytes took an intermediate position. It was also clear that the response is directly related to the dose of irradiation given and, therefore, to the number of residual stem cells. This also explains, for instance, why the effectiveness of GM-CSF to stimulate thrombocytes may vary in various clinical trials. These data were compared to the blood cell regeneration rates following autologous transplantation of graded numbers of bone marrow cells to assess the numbers of residual hemopoietic stem cells following TBI without bone marrow transplantation (Table I). The data were fitted to a mathematical description relating GM-CSF effectiveness to residual numbers of hemopoietic cells. This relationship can be used to interpret clinical data on the use of hemopoietic growth factors following TBI.

The data also showed, that the early response to GM-CSF is related to the number of residual hemopoietic stem cells and, therefore, to the irradiation dose received. As such, it may serve as an indicator for the need of bone marrow transplantation following accidental exposure to radiation. We therefore further analyzed the time point after TBI at which the GM-CSF response can be used to assess the radiation damage. For that purpose, the effects of GM-CSF on the cumulated white blood cells (WBC) were also analyzed for shorter observation periods. During the fist five days following TBI, the white blood cells decrease, and the cumulated white blood cells were not related to the radiation dose. Although treatment with GM-CSF resulted in an increase of the cumulated WBC during this episode, this effect was not related to the dose of TBI. Hence the cumulated white blood cells during the first five days following TBI cannot be used for biological dosimetry. In GM-CSF treated monkeys, the cumulated leukocytes in the period between day 6 and day 8 were correlated weakly to the dose of TBI. This was not the case in the control group. Therefore, the cumulated leukocytes in the period between day six and day eight, allow for only a very rough estimate of the dose of TBI (Table II). Longer observation periods (day 6 - 15 or day 6 - 22) result in a slight increase of the accuracy of the dose estimate. Without GM-CSF, the cumulated cell counts can only be used for biological dosimetry following 3 weeks of observation, and the estimate of the irradiation dose is even less accurate.

The results are summarized in Table II. A full evaluation awaits studies with IL-3, which acts on a developmentally more early cell type than GM-CSF, and on studies in which the synergism between IL-3 and GM-CSF or other growth factors can be explored in vivo. GM-CSF given before irradiation had no beneficial effect.

Human recombinant interleukin-3 is considerably less effective on the BM cells of Macaca species, including rhesus monkeys, than on human or chimpanzee BM cells. Hence, the molecular cloning of the gene encoding rhesus monkey IL-3 was accomplished. 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 the rhesus IL-3. The rhesus IL-3 gene was expressed in Bacillus licheniformis for large-scale production and is currently available. Using this material and similarly expressed human IL-3, we indeed established that the affinity of human IL-3 for the bone marrow IL-3 receptor is about 50-fold lower than that of rhesus monkey IL-3. Recombinant rhesus monkey IL-3 was administered to normal rhesus monkeys in graded doses ranging from 3 to 30 microgram/kg/day subcutaneously for 30 consecutive days or given as a continuous intravenous infusion at a dose of 30 microgram/kg/day for 16 days. Following a lag phase of about one week, a highly increased, dose dependent production of bone marrow derived blood cells was observed, preceded by amplification of bone marrow hemopoietic progenitor cells. Simultaneously, peripheral blood progenitor cells rose. The increases included white cells (monocytes and the three types of granulocytes, Figure 2), the red cell series (normoblasts and reticulocytes) and platelets. The continuous intravenous route of administration proved to be more effective than subcutaneous administration, even if given in multiple dayly injections. In the monkey which received IL-3 by continuous infusion, white cells in the second week of treatment rose to about 75 x 10^9/l, while the administration had to be discontinued because of severity of side effects at day 16. Characteristically, a peripheral blood T lymphocyte response was absent. Neither absolute cell numbers nor the variety of cell types produced were precedented in studies with human IL-3 in Macaca species. Analysis of nucleated blood cells revealed, apart from vast increases in numbers of eosinophilic and neutrophilic granulocytes, monocytes and normoblasts, the appearance of large quantities of cells designated as atypical (basophilic) granulocytes, also reported in studies with human IL-3 in Macaca species. Accordingly, intracellular histamine levels of peripheral blood cells rose directly proportional to the numbers of these atypical basophils. Dose related side effects included urticaria starting around day 10 of treatment, prominent axillary and inguinal lymph nodes from day 20 onward, and at high IL-3 doses, facial and scrotal oedema starting in the third week of treatment. The skin lesions were most numerous and prominent in monkeys which received 30 microgram/kg/day IL-3. At such high doses of IL-3, thrombocytopenia

developed, contrasting thrombocytosis at lower doses. Thrombocytopenia could not be explained from decreased production, since megakaryocytes in bone marrow and shift platelets in blood were abundant. Thrombocytopenia was coincident with vasculitis and hemorrhage in the generalized skin lesions of the monkeys given IL-3 in high doses. Withdrawal of IL-3 not only prompted spontaneous resolution of skin lesions, but also of thrombocytopenia and other side effects without any sequeles within two to four days. Recent sudies showed that the side effects can be almost completely prevented by simultaneous administration of histamine H1 antagonists without affecting the bone marrow response.

Such results demonstrate that in vivo administration of homologous IL-3 to rhesus monkeys resulted in a dose-dependent stimulation of the production of all bone marrow derived cell lineages. In line with existing in vitro data and strongly supported by bone marrow analyses, this may be simply explained by increased production of progenitor cells from developmentally early, multipotential bone marrow cells resulting in augmented production of all bone marrow derived blood cell lineages. It may now be predicted that many of the effects of IL-3 can be selectively amplified if combined with lineage specific growth factors, such as erythropoietin for red cells and G-CSF, M-CSF or GM-CSF for cells of the granulocyte/monocyte series. Selective amplification of the response by other hemopoietic growth factors might well provide a most versatile use of IL-3 and may reduce effective single therapeutic doses of IL-3 and lineage-specific hemopoietic growth factors to doses that more approach physiological requirements, thus reducing side effects. Currently, IL-3 is being tested in irradiated monkeys similar to the study completed for GM-CSF.

II. Expanding stem cell populations in vitro

Extensive studies in mice have shown the possibility of stimulating murine repopulating stem cells with IL-3 and expand them about 3 to 5 times, thus facilitating engraftment of autologous as well as allogeneic bone marrow cells. In mice, we are in the process of identifying the non-CFU-S population that is responsible for hemopoietic reconstitution of irradiated recipients and of

characterizing its HGF responses. Since the same HGFs are now available for rhesus monkeys as well as humans, the mouse experiments provide guide lines for experiments on primate bone marrow. Preluding on the primate experiments with growth factors, we have developed a simple, rapid and large-scale method for positive identification and purification of rhesus monkeys and human stem cells and progenitor cells. The high avidity non-cytotoxic mouse IgG2a anti human CD34 monoclonal antibody ICH3 reacts with the HPCA-1 molecule and recognizes a similar epitope on rhesus monkey bone marrow (BM) cells. In both species approximately 1-4 % of the BM cells are CD34 (ICH3) positive. From the regeneration rate of lethally irradiated rhesus monkeys transplanted with only 2 x 10^5 autologous ICH3+ cells/kg body weight, it was concluded that the repopulating stem cells were 40- to 140-fold enriched. To develop a method for large scale separation on this basis, Protein A was covalently bound to immunomagnetic beads and ICH3 was conjugated to the Protein A. Competitive elution of cells binding to ICH3 was done by excess soluble IgG. The procedure may also serve as a basis for positive selection of hemopoietic stem cells for autologous as well as allogeneic bone marrow transplantation. Approximately 1% of the original nucleated cell count was recovered as rhesus monkey ICH3+ cells and contained on the average 60% - 70% of the hemopoietic progenitor cells; this compares very well with methods based on negative selection of stem cells by eliminating unwanted cells. The fraction of residual T lymphocytes was less than 1 % of the original BM. Scatter plots of whole bone marrow versus $CD34^+$ cells isolated in this way are shown in Figure 3. Pilot experiments using stimulation of such purified cell suspensions with relatively low concentrations of rhesus monkey IL-3 demonstrated a rapid expansion of GM-CSF responsive progenitor cells and the current availability of rhesus monkey IL-3 will allow us to complete these studies. Although prolonged stimulation of murine stem cells with mouse IL-3 has been shown to result in large production of committed progenitor cells but a concomitant loss of repopulating stem cells, and, therefore, success in primates is not guaranteed, we will study varying time schedules of IL-3 incubation in vitro, using hemopoietic regeneration rate following autologous transplantation in high dose irradiated monkeys as an end-point. Since novel hemopoietic growth factors, notably SCF (stem cell factor), have

recently been identified, it is not excluded that other growth factors than those that are currently being developed for pharmaceutical purposes, will be suitable for the purpose of expanding stem cell populations.

The $CD34^+$ cells suspensions produced in this way have meanwhile been tested for capacity of autologous reconstitution. The experiments showed, that as little as 2 x 10^5/kg $CD34^+$ cells reconstitute as well as 10^7/kg whole bone marrow cells. In addition, allogeneic experiments demonstrated that the $CD34^+$ cells reconstitute myeloid as well as lymphoid lineages.

SUMMARY

The present work has led to a reappraisal of the radiation sensitivity of hemopoietic stem cells of primates. If the obtained data can be extrapolated to humans, intensive supportive care rather than bone marrow transplantation is the treatment of choice for victims of high doses of accidental irradiation, since residual numbers of stem cells can be expected to ultimately regenerate the blood-producing system even at doses of 10 Gy TBI (the dose at which irreversible gastrointestinal damage becomes the most important acute radiation effect).

The research work done has further given clues about the use of hemopoietic growth factors. Tested were the possibility that the early response to GM-CSF can be used as a parameter for the number of residual stem cells and, therefore, for the dose of irradiation received, as well as the efficacy of this growth factor in shortening or preventing the period of leukopenia. Although a relationship between the early GM-CSF response and the dose of irradiation could be established, the variability of the response and the time required for a response did not support the idea that GM-CSF could be used practically for this purpose. However, GM-CSF was fully effective in preventing leukopenia after a dose of 5 Gy TBI (an about 2-log stem cell depletion). It became increasingly less effective at higher doses of irradiation and was clinically ineffective after a dose of 8 Gy TBI (an about 3-log stem cell depletion). To enable studies with the much more broadly acting growth factor IL-3, it was necessary to clone and express the gene encoding rhesus monkey IL-3 and to produce the

protein, since the species specificity of human IL-3 made studies in rhesus monkeys with the human growth factor unreliable. During the contract period, the molecular cloning and production of rhesus monkey IL-3 was successfully achieved and preclinical pilot studies, using unirradiated monkeys, have been completed.

The practical feasibility of in vitro expansion of hemopoietic stem cells requires a rapid and reproducible method to concentrate stem cells. We endeavoured the positive selection of hemopoietic stem cells by developing a large-scale method based on selection of $CD34^+$ cells. The method enables the selection of hemopoietic stem cells at any scale within a matter of hours, yielding preparations that contain about 60 - 70% of the original stem cell content of bone marrow concentrated to about 1% of its original cellular content. These preparations have been successfully tested for autologous transplantation experiments and were shown in allogeneic experiments to be capable of hemopoietic regeneration of along myeloid as well as lymphoid regeneration. The availability of this method as well as the availability of rhesus monkey IL-3 will enable us now to study the option of stem cell expansion in vitro in detail.

Table I GM-CSF EFFECTIVENESS RELATED TO THE IRRADIATION DOSE

cell type	significant effect observed		no effect expected at all	
	dose of TBI	corresponding fraction of surviving stem cells	dose of TBI	corresponding fraction of surviving stem cells
leukocytes*	<8 Gy	3×10^{-4}	10 Gy	5×10^{-5}
thrombocytes	<7 Gy	10^{-3}	9.5 Gy	7×10^{-5}
reticulocytes	<6 Gy	3×10^{-3}	7 Gy	10^{-3}

* representing neutrophils, eosinophils and monocytes

Table II BIOLOGICAL DOSIMETRY BASED UPON THE EFFECT OF GM-CSF ON CUMULATED WBC FOLLOWING TBI

observation period (days)	dose (Gy)	GM-CSF cumulated wbc (calculated)	GM-CSF dose range (95% conf lim)	PLACEBO cumulated wbc (calculated)	PLACEBO dose range (95% conf lim)
6 - 22	4	112	<5.0	21	<5.6
	6	68	4.6 - 7.1	16	<7.2
	8	28	>6.9	11	>6.4
6 - 15	4	61	<5.1	10	
	6	30	4.9 - 7.0	8	
	8	0	>7.0	5	
6 - 8	4	21	<5.1	3	
	6	10	4.8 - 7.0	3	
	8	0	>6.8	2	

wbc = white blood cells

Legends to Figures

Figure 1.

The three putative mechanisms by which hemopoietic growth factors may mitigate cytopenia following cytoreductive irradiation, here indicated for therapeutic purposes, but the same principles also applying to accidental high dose total body irradiation. Upper panel: pretreatment with HGFs resulting in a high peripheral blood cell count and (hypothetically) an increase in stem cell numbers leading to a later onset of and an earlier recovery from cytopenia. Lower panel: accelerated reconstitution by treatment with HGFs.

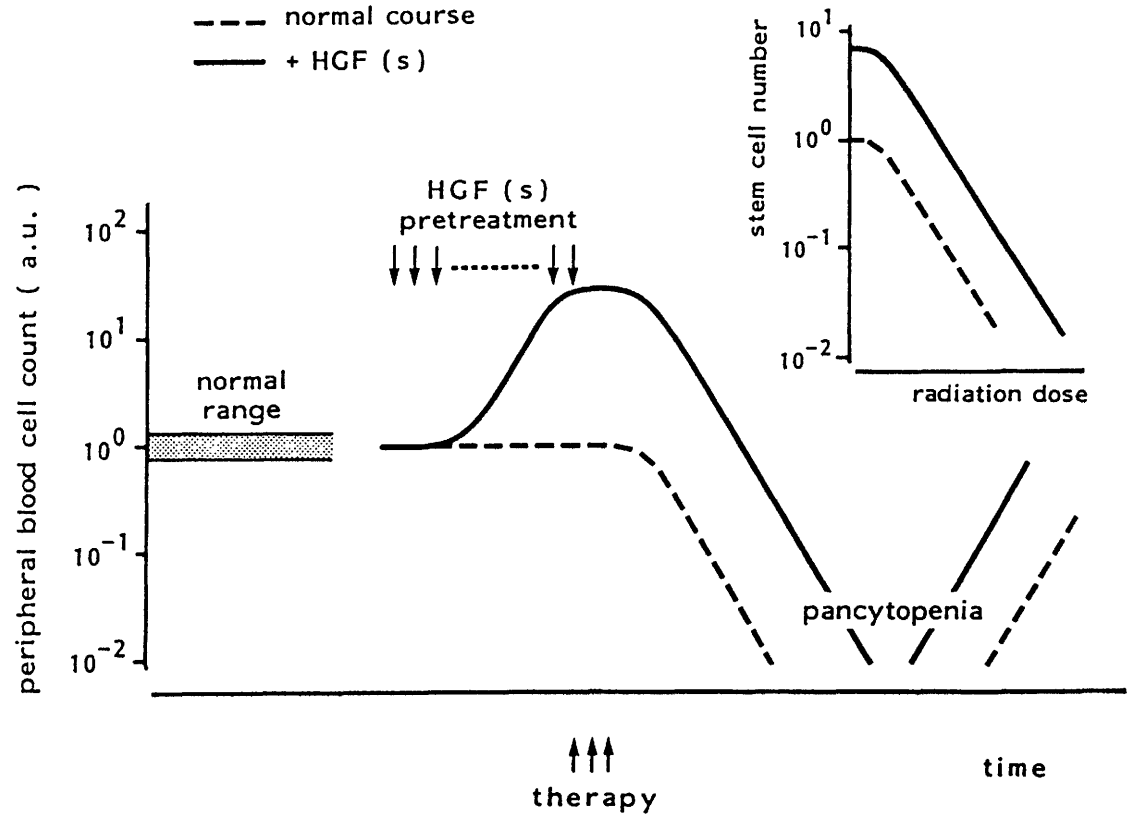
Figure 2.

Peripheral nucleated blood cell counts following administration of graded doses of Rh-IL-3, from top to bottom panels: continuous infusion of 30 microgram/kg/day during 16 consecutive days, and daily single subcutaneous injection of 30, 10 and 3 microgram/kg/day, respectively, given for 30 consecutive days and a simultaneous control monkey, which did not receive IL-3, but was otherwise treated identically. The differential counts were cumulated, from top to bottom: normoblasts, eosinophilic and neutrophilic granulocytes, neutrophilic bands, atypical granulocytes, monocytes and lymphocytes. Note scale difference between upper panel and the other panels.

Figure 3.

Scatter plot of forward and perpendicular light scatter showing the selected $CD34^+$ bone marrow cell population in comparison to whole bone marrow of rhesus monkeys. The population shown in the lower panel contains 60 - 70% of hemopoietic stem cells and progenitor cells, with cell numbers reduced to about 1% of the original bone marrow. E, L, G and B stand for respectively erythrocytes, lymphocytes, granulocytes and blast cells.

FIGURE 1


normal course
+ HGF (s)
peripheral blood cell count (a.u.)
10^2
10^1
10^0
10^{-1}
10^{-2}
HGF (s)
pretreatment
normal
range
stem cell number
10^1
10^0
10^{-1}
10^{-2}
radiation dose
pancytopenia
therapy
time

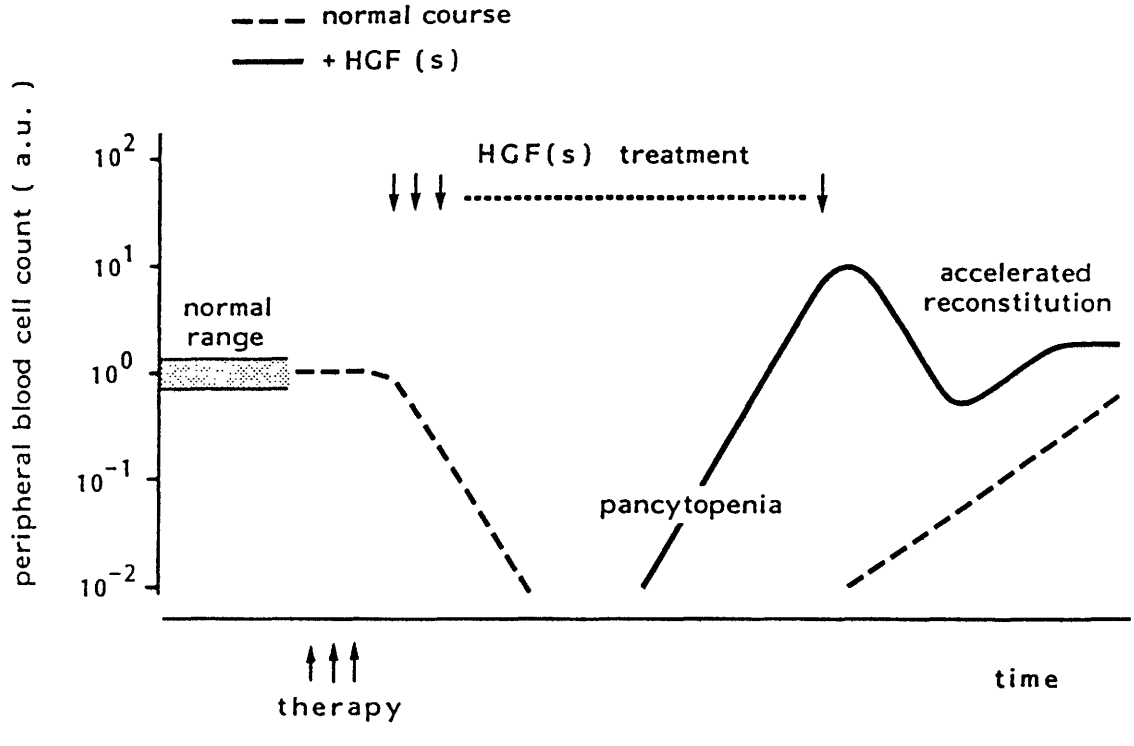

normal course
+ HGF (s)
peripheral blood cell count (a.u.)
10^2
10^1
10^0
10^{-1}
10^{-2}
HGF(s) treatment
normal
range
accelerated
reconstitution
pancytopenia
therapy
time

FIGURE 2

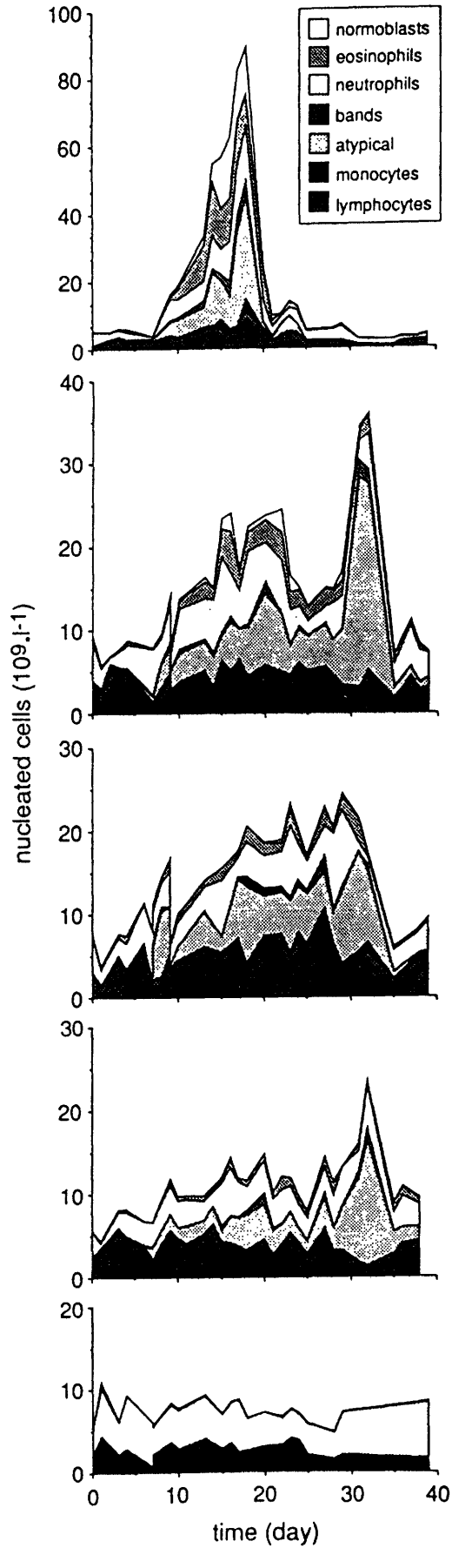
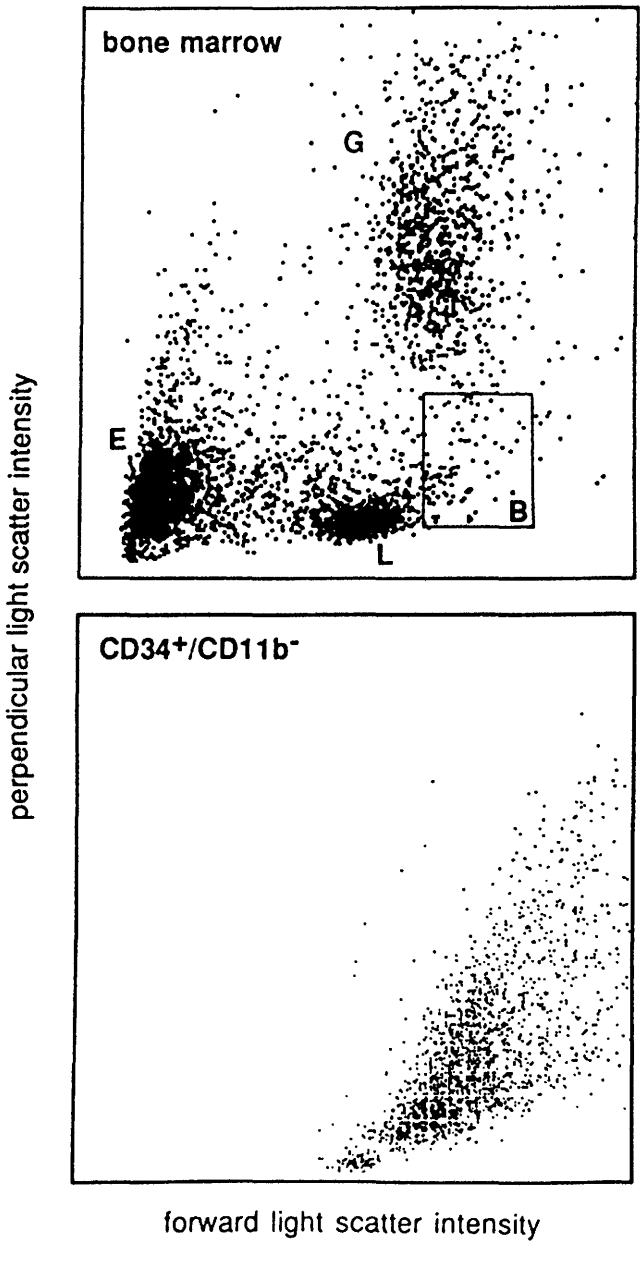

normoblasts
eosinophils
neutrophils
bands
atypical
monocytes
lymphocytes
nucleated cells (10⁹.l⁻¹)
time (day)

FIGURE 3


bone marrow
G
E
B
L
CD34+/CD11b-
perpendicular light scatter intensity
forward light scatter intensity

UNIVERSITÄT ULM

INSTITUT FÜR ARBEITS-UND SOZIAL MEDIZIN

CONTRACT BI6 - 0289 - D

THE ACUTE RADIATION SYNDROME: A CHALLENGE FOR THE MEDICAL PROFESSION IN CASE OF RADIATION ACCIDENTS

T. M. FLIEDNER

1. Introduction

It is the purpose of this report to describe the scientific work that has been conducted within the "Post-Chernobyl-Programme" of the European Communities between March 1, 1988 and February 28, 1990 by the Radiation Medicine Research Group of the Department of Clinical Physiology, Occupational and Social Medicine of the University of Ulm. This group concentrated its efforts on the "Acute Radiation Syndrome", its pathophysiology as well as its diagnostic and therapeutic implications. The questions to be answered by the research group may be stated as follows:

1. What observations were published about the acute radiation syndrome as a consequence of the Chernobyl-accident?

2. What are similarities and dissimilarities in comparison to other radiation accidents since 1945?

3. What are the principle pathophysiological mechanisms leading to the acute radiation syndromes observed after accidental whole body exposure?

4. Which are appropriate diagnostic indicators to predict course and outcome of the acute radiation syndromes?

5. What is known about possibilities and limitations to appropriate take care of persons suffering from the acute radiation syndrome?

6. What are consequences out of the analysis of the "state of the arts" for further research work in order to improve existing and develop new strategies for medical radiation accident management in Europe?

The efforts of the Ulm research group represent only part of the work carried out by European research laboratories in the "Post-Chernobyl-Programme". Through a series of meetings with the other groups it was possible to complement the different activities. It also became obvious, that it is desirable if not essential, to form a "European Research Network in Radiation Accident Medicine" in order to improve the scientific basis for radiation accident management and to develop strategies to cope with radiation accident situations of different degrees of complexity from the medical viewpoint.

2. Acute radiation syndrome observations in the Chernobyl accident

On April 26, 1986 a severe radiation accident occured in the Chernobyl-Atomic-Power-Plant, the health consequences of which are still being explored even today, 4 years later.

In August 1986, a conference was held at the International Atomic Energy Agency in Vienna (IAEA) in order to inform the scientific public about the event. In the proceedings of this conference, Annex 7 describes the medical-biological problems. It is entitled: "Data on operational and emergency staff of the nuclear power plant who were exposed to radiation: size of doses received and the consequences for health. Experience of treatment" (1).

This report allowed to obtain information on the signs and symptoms of 203 exposed persons who developed the "acute radiation syndrome". This material served as an important input for the further research work carried out at the University of Ulm in an attempt to review "the state of the arts" and to come to recommendations about the possibilities and limitations in the European Community to appropriately handle radiation accidents should they occur in the future from the medical perspective.

In Table 1, the major signs and symptoms are listed as observed in 203 persons that received a total body radiation exposure in the very early phase after the radiation accident. These persons were classified into four groups suffering in different degrees from an acute radiation syndrome. 105 persons showed only a mild syndrome. There were only a few hematological changes, but there was neither a gastrointestinal symptomatology nor skin lesions. None of these persons died within the first few months after

Hämatologische Form des akuten Strahlensyndroms/Tschernobyl

(UdSSR-Bericht IAEA-Konferenz Wien, Aug. 1986)

	Grad IV.	Grad III.	Grad II.	Grad I.	Σ
Zahl der Patienten	22	23	53	105	203
Vermutete Dosis (Gy)	) 6 (bis 16)	4.2 - 6.3	2-4	0.8-2.1	
Allgemeine Symptome (Übelkeit, Erbrechen)	+++	++	+	±	
Hämatolog. Syndrom	+++	++ bis +++	+ bis ++	0 bis +	
Gastroint. Syndrom	+++ 7-9 d	++	±	0	
Verbrennungen der Körperoberfläche	+++ (22/22) 40-90%	++ (6/23)	keine	keine	
Todesfälle	19* 4-50 d.	7 14-49 d.	keine	keine	26*?

* 2 Frühsterbefälle nicht berücksichtigt

+++ schwer bis extrem
++ mittelschwer
+ mäßig bis leicht
0 fehlt

Table 1

exposure. The estimate dose was 0.8-2.1 Gy. It is known from previous analysis, that such doses would be far below the LD50/60 days and would not require a special therapy. 53 persons were classified into a group designated as "grad II". In these patients, there was a general symptomatology with nausea and vomiting but of a mild type. It was reported that these patients showed some nausea and vomiting, blood cell changes of a mild to significant degree, some alterations of the gastrointestinal tract but no skin lesions. There were no deaths in this group. The radiation dose estimates ranged from 2-4 Gy. A total of 23 patients were assigned to the group "grad III". It is noteworthy that there were 7 casualties in this group with death occurring between day 14 and 49. This fact, together with the signs and symptoms - significant nausea and vomiting, blood cell changes and a gastrointestinal symptomatology - indicate a "hematological form of the acute radiation syndrome. There were severe skin burns in 6 of 23 patients. It is quite

possible that these burns were the major reason for the fatal outcome in these cases and not the hematological and gastrointestinal alterations. A total of 22 patients were assigned to the group "grad IV". 19 of them died between 4-50 days. There were intensive evidence for nausea and vomiting early after the accident. There were also severe blood cell changes and a gastrointestinal symptomatology between day 7-9 after exposure. All of them had extensive skin burns (40-90% of the body surface). The calculated dose was in excess of 6 Gy perhaps going up as high as 16 Gy.

It has been of interest to analyse the signs and symptoms of these patients with an acute radiation syndrome in some more detail (Table 2).

Klassifizierung des akuten Strahlensyndroms nach Schweregrad/Tschernobyl
(UdSSR-Bericht IAEA-Konferenz Wien, Aug. 1986)

Symptome	Grad IV	Grad III	Grad II	Grad I
Primärreaktion (Übelkeit, Erbrechen)	< 30 Min.	30-60 Min.	1-2 St.	>2 St.
Latenzperiode (bis zum Höhepunkt der Reaktion)	6-8 d.	8-17 d.	15-25 d.	>30 d.
Hämatologie				
Lymphozyten/mm^3	< 100 3-6 d.	100-200 3-6 d.	300-500 3-6 d.	600-1000 3-5 d.
Granulozyten/mm^3	~ 500 7-9 d.	< 1000 8-20 d.	> 1000 20-30 d.	3000-4000 8-9 d.
Thrombozyten/mm^3	< 40.000 8-10 d.	< 40.000 10-16 d.	> 40.000 17-24 d.	40.000-60.000 25-28 d.

Table 2

As can be seen, there is a correlation between the primary reactions (nausea and vomiting) and the extend of radiation exposure: the earlier the onset of this symptomatology, the

higher the exposure dose. Also, the time between the maximal reactions of the organism and the exposure is the shorter, the higher the exposure dose has been. The hematological parameters indicate, that the patients of the group "grad IV" suffered from gastrointestinal form of the radiation syndrome with little if any chance of hematopoiesis to recover spontaneously. The patients classified into the group grad IV showed a symptomatology pattern, compatible with a severe form of the hematological radiation syndrome, while those classified into the group "grad II" suffered from a milder hematological form. The blood cell changes in persons assigned to group "grad I" were obvious but at no time was there any evidence for an irreversible damage to the blood cell production.

Unfortunately, there has not been - so far - a detailed report on the clinical signs and symptoms of all these 203 accident victims. However, it will be of great importance for the future to compare the clinical course and outcome in these patients to the course and outcome of patients in other radiation accidents. It is only then that one will begin to understand similarities and differences and whether the skin burns in the Chernobyl cases contributed in what way to the outcome.

However limited the information may be, it is of interest to at least compare 2 reported cases (August 1986, IAEA conference) (1) with the hematological data in our accidents.

In figure 1, the granulocyte changes are depicted for the 2 reported Chernobyl patients in comparison to patients A and C of the Oak Ridge accident of 1958 and to persons exposed in March 1954 on the Marshall islands. In figure 2, the data are given for the platelets.

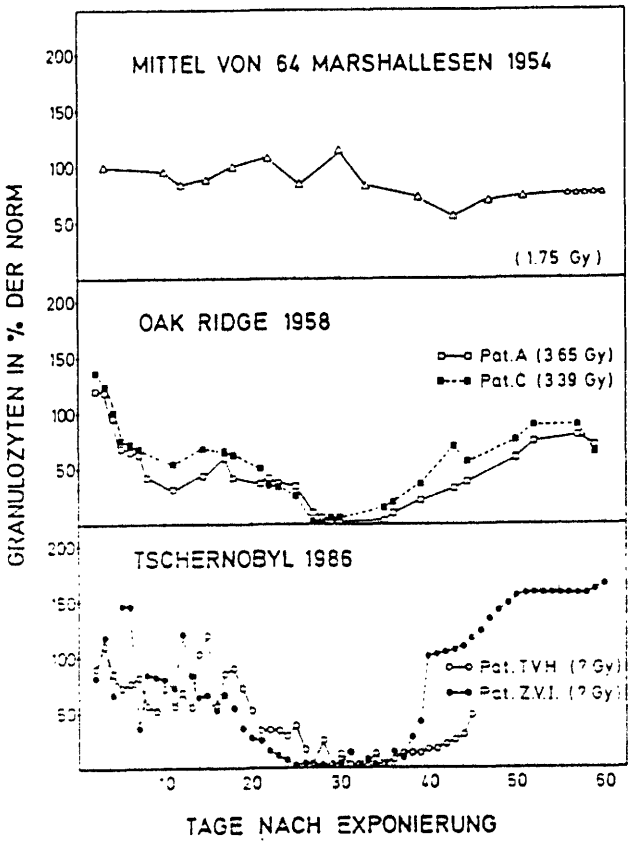


Figure 1

As far as the granulocyte changes are concerned (fig. 2) it is obvious, that in the 2 Chernobyl and in the 2 Oak Ridge patients, there was in all cases an "abortive rise" between days 8-18 after exposure and a nadir of values on or around day 28-30. This course of events clearly indicates that in these instances there was a spontaneous granulocyte recovery which means that the damage to the stem cell pool was reversible (see later). Thus, one would expect that the exposure dose to the 2 Chernobyl patients must have been similar to that calculated for the Oak Ridge patients.

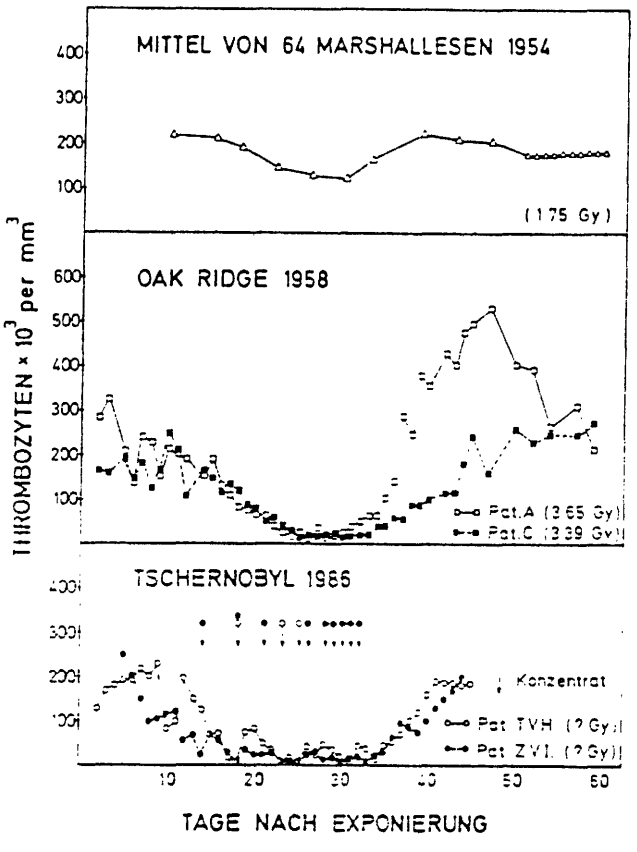


Figure 2

These conclusions are also born out by the observations regarding the platelet count. Some reservations must be expressed with respect to the platelet numbers because it was not stated, which counting method had been employed. However, the main observation in that the platelets reached their lowest level between day 20 and 30 which is support for the notion, that the megakaryocyte system did not cease to support platelet production and that there was a spontaneous platelet recovery beyond day 30. These patients were treated by platelet transfusions in order to overcome the consequences of thrombocytopenia. In Oak Ridge, such measures were not considered to be necessary. It may well be that the biologically effective dose in the Chernobyl patients was a little higher than in Oak Ridge but probably

not more than 10-20%. In any event, the granulocyte as well as platelet pattern is indicative of a reversibility of the hemopoietic damage requiring only "replacement" therapy as described earlier (2).

The similarity in the clinical course of the 2 reported Chernobyl cases and the Oak Ridge patients is also born out by the analysis of early lymphocyte changes (Fig. 3).

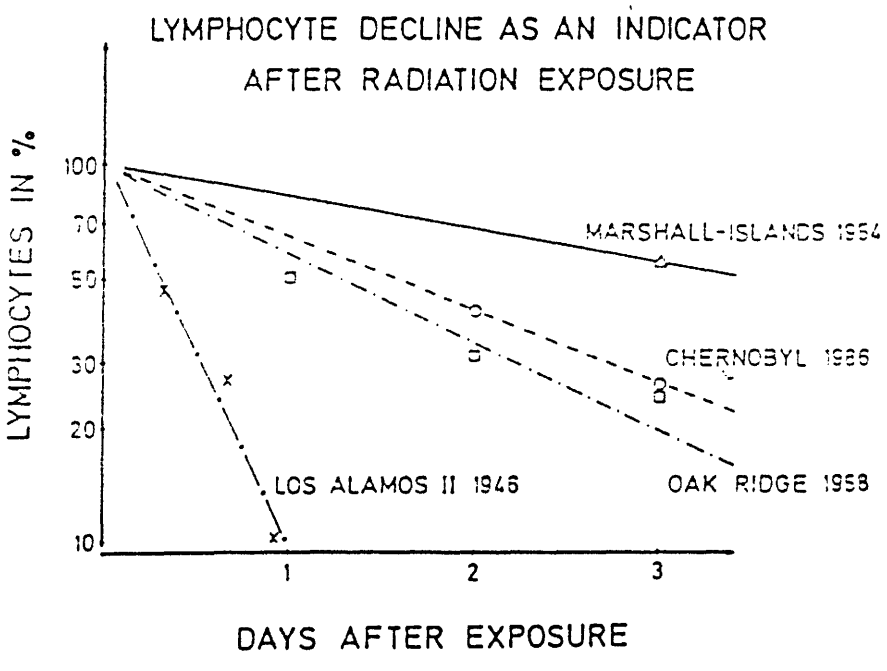


Figure 3

It can be seen, that the lymphocytes in the Chernobyl and the Oak Ridge cases decreased to 30-40% of normal within 2 days after exposure. This pattern is distinctly different from the case "Los Alamos II" who was exposed to about 12 Gy, a dose causing a severe lymphopoiesis (down to 10% of normal) within 24 hours.

These studies lead to the conclusion, that it would be of great importance for radiation medicine research to try to obtain as much information as possible on the actual clinical observations in radiation accident victims in order to compare one to the other and to extract from all

observations the key differences and similarities. These data would then form the basis for a further pathophysiological analysis in order to study the mechanisms of the radiation syndrome and to derive suggestions for an improvement of existing and the development of new approaches to handle such patients in the most effective way.

3. Establishemnt and utilization of a data base for radiation accident victims

In the course of this "Post-Chernobyl-Project" it was considered necessary to try to establish a data base of as many as possible radiation accidents since 1945. The goal was to register all accidents that resulted in whole body exposurees with penetrating ionizing radiation and that lead to acute radiation syndromes of different degrees of clinical severity. For each person involved, an attempt is made to collect as many clinical signs and symptoms as possible and to try to correlate hematological and other changes to the course of disease and its outcome in order to establish a series of "biological indicators" that are of value to predict early after exposure (i.e. within 1-3 days) the most likely clinical course and to establish the most optimal therapeutic concepts.

Radiation Accident No.	Year of Accident	Type of Radiation	Number of Persons Involved	Number of Persons Evaluated	Number of Deaths	References
1. Los Alamos I	1945	Gamma, n	2	2	1	23
2. Los Alamos II	1946	" "	8	7	1	23
3. Argonne	1952	" "	4	4		6,22
4. USSR	1953	" "	?	2		20
5. Marshall Islands	1954	Fallout	290	267		1,9
6. Oak Ridge	1958	Gamma, n	8	8		7,15
7. Vinca	1958	" "	8	6	1	26,27,32
8. Los Alamos III	1958	" "	11	2	1	37
9. Lockport	1960	X-rays	9	2		12,24,25
10. Madison	1960	Gamma	12	1		36
11. Hanford	1962	" n	22	1		11
12. Puerto Rico	1962	"	7	4		8
13. Wood River Junction	1964	" n	5	1	1	30
14. Mol	1965	" "	1	1		3,28,35
15. New Yersey	1978	"	1	1		2
16. Tennessee	1975	"	1	1		43,44
17. Brescia	1975	"	1	1	1	28,35
18. New Yersey	1977	"	1	1		2
19. China 4	1980	"	1	1		16,17
20. Norway	1982	"	1	1	1	38
21. Chernobyl	1986	" n + Fallout	203	5	28	19,33
22. China 6	1986	Gamma	2	2		16,17
			598	321	35	

Table 3

In table 3, the present state of the global radiation accident data base is summarized. The research group in Ulm was able so far to collect information on 22 accidents between 1945 and 1986 resulting in acute radiation syndromes in 598 persons. The most important type of radiation involved was gamma radiation alone or in combination with neutron irradiation. In a few cases, the radiation was produced in a X-ray tube. The inhabitants of the Marshall Islands and the Chernobyl victims were also exposed to radiative nuclids resulting in contamination of the skin and/or incorporation. Of the 598 persons involved, 35 died (5.8%) as a direct consequence of the exposure. It will be of interest to try in the future to find out what happened later (1-30 years) to these exposed persons and what was the pattern of health impairments developing as "late effects". A more extensive clincial and hematological evaluation has been performed so far in 68 of 321 registered individual case histories. It became obvious, that it will not be possible to collect detailed information on many of the patients. However, in collaboration with the global scientific community, it might well be possible to extend the present analysis to more cases. In addition, although a lot of information has been collected on the 290 individuals exposed in 1954 on the Marshall-Islands (thanks to the collaborative efforts of scientists at the Brookhaven National Laboratory), these data are not yet discussed in this report since most of them belong to the exposure category I (low exposure) and their evaluation does not add much to the present report on indicators for the management of the severe acute radiation syndrome.

In this report, it appears of greatest interest to analyse the case reports registered in the data base with respect to changes of neutrophils, platelets and lymphocytes in the course of the response to whole body irradiation. The analysis was performed with the question in mind, whether

these changes would help to answer the decisive question that a physician needs to answer: is the damage to the hemopoietic stem cell pool likely to be reversible or not, does the patient require replacement or substituion therapy (see 2).

It was possible to form 6 exposure categories on the basis of blood cell response patterns. This assignment of patients to "hemopoietic response categories" was done by plotting for each patient his own granulocyte, platelet and lymphocyte changes as a function of time after radiation exposure up to 60 days.

CLASSIFICATION	INITIAL GRANULOCYTOSIS		FIRST MINIMUM		HEIGHT OF ABORTIVE RISE		NADIR		DURATION OF ABORTIVE RISE	RECOVERY
N. Pat.	Count% Mean STD Range	Day Mean STD Range	Count% Mean STD Range	Day Mean STD Range	Count% Mean STD Range	Day Mean STD Range	Count% Mean STD Range	Day Mean STD Range	Day Mean STD Range	Day Mean STD±ERR Range
CATEGORY II 40	167 ±69 71-413	2 ±1 1-6	36 ±17 21-92	11 ±3 5-19	98 ±22 51-156	19 ±3 11-30	31 ±9 17-50	39 ±6 21-66	28 ±8 11-56	60 ±1 21-63
CATEGORY IIB 10	176 ±69 111-313	2 ±2 1-6	30 ±13 11-76	10 ±2 7-12	100 ±38 46-196	16 ±6 11-24	8 ±3 1-18	30 ±6 21-39	20 ±8 11-31	60 ±3,2 21-66
CATEGORY IV 9	200 ±77 125-339	1 ±1 1-2	22 ±13 11-48	9 ±2 7-11	60 ±9 21-96	13 ±3 11-16	0,3 ±0,3 0-1	27 ±2 21-32	18 ±3 11-23	33 ±3,7 11-61
CATEGORY V 3	193 ±48 146-242	1 ±2 1-3	6 ±1 5-7	7 ±1 6-8						
CATEGORY VI 2	919 ±412 628-1213	1 0 1								
p	0.001	0.1	0.001	0.01	0.001	0.001	0.001	0.001	0.001	

Table 4

Table 4 shows the results of a first analysis of the granulocyte response pattern in 68 patients exposed in radiation accidents to whole body irradiation. The first "indicator" is the "initial granulocytosis". With this is meant the increase of granulocytes in the first 2-3 days after exposure significantly above the normal level. It is

of great interest to note, that the most intensive granulocytosis was observed in 2 patients that received radiation doses in excess of 20-30 Gy and died within 3 days of exposure ("CNS-Syndrome"). In these patients the granulocytosis reached values of more than 900% within 24 hours. In the response categories II to V it was always possible to observe an initial granulocytosis within 1-2 days with values of 167 to 200% of normal. This initial granulocytosis has been recognized as an important parameter since 1955 when our group established its pathophysiological mechanisms and termed it as a "stress-reaction" of the bone marrow caused by shift of granulocytes from marginal to circulating pools and from bone marrow into blood as a consequence of sinusoidal reactions of the marrow microcirculation.

A second "indicator" of importance and of pathophysiological significance is the first maximum of granulocytes. In table 4 it is of interest, that there is a distinct difference between patients in category IV and V. In the latter, the neutrophil count drops to 4% of normal within 7 days. Actually, the major drop occurs between day 4 and 6 due to the fact, that the maturation time since the last myelocyte division is for granulocytes (T 1/2 = 7 hours in blood) 4-5 days. If all dividable precursors in the marrow are radiation injured then no newly produced granulocytes enter the blood after 4-5 days so that the steep drop in granulocyte counts is expected to occur at this time. On the other hand, if somewhere in the body some myelocytopoiesis and hence granulocyte productions is left, the extend of the first granulocytopenia after exposure will not be excessive. This explains that the first minimum for patients in categories IV, III and II reaches only 22, 50 and 54% of normal at days 9, 10 or 11 respectively. The extend of this first granulocyte minimum between 5 and 10 days can be considered of utmost importance in trying to answer the

question, whether a spontaneous recovery of hemopoiesis (and of the patient) is likely or unlikely.

A third indicator is the height of the abortive rise. The higher the damage, the lower is this abortive rise. There is a distinct difference between category IV on the one hand and categories II and III on the other. This corresponds actually to the appearance of the granulocyte nadir in these categories: the more extensive the damage, the shorter the duration of the abortive rise and the earlier the nadir which - in cateogry IV - is 27 days.

The pathophysiological mechanisms behind this pattern is depicted in figure 4.

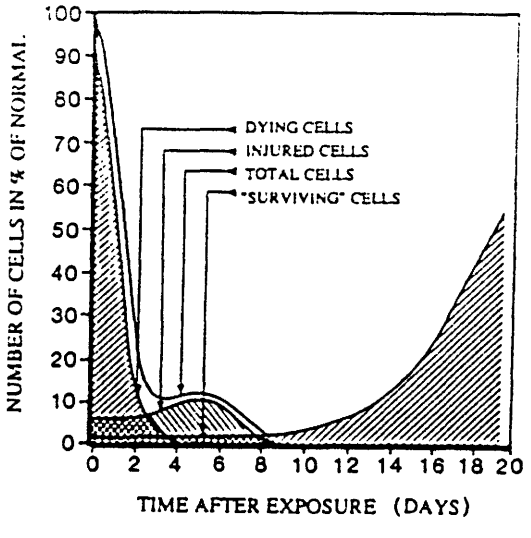


Figure 4

This concept was first developed by our group in 1965 (2) and has now been used to develop a biomathematical computer model to simulate the granulocyte pattern after total body exposure and to calculate the "remaining stem cell units" in the bone marrow from which a spontaneous recovery of hematopoiesis could commence.

This concept establishes the hypothesis, that total body exposure results in the damage of pluripotent stem cells in the body whereever in the skeleton they might be localized. The exposure will result in a damage in accordance with a D_o of about 80 to 100 cGy. There will then be stem cells remaining from which a recovery could commence. If there are unirradiated or repaired stem cells ("surviving cells") then they would be the origin of the eventual recovery of the system. The biology of total body irradiation shows, however, that the response to radiation can only be explained if one assumes a further category of stem cells, the so called injured stem cells. They would not any longer have the potential of unlimited replicative potential but oof limited potential. It is this group of stem cells that is responsible foor an "abortive" recovery. This abortive recovery is the less pronounced and the shorter, the lower the number of the "injured stem cells" is. This hypothesis would lend support to the idea, that the abortive rise phenomenon could be used in the evaluation of radiation exposed individuals.

The radiation accident data base so far established provided the opportunity to also analyse in detail the significance of lymphocyte changes as an indicator for radiation exposure.

The lymphocyte pattern was evaluated in 55 patients suffering from an acute radiation syndrome. The questions to be asked were as follows: Are these characteristic changes in lymphocyte counts during the observed postaccidental period? If yes, do patterns exist which are comparable to the known granulocyte patterns? For each person analysed, the lymphocyte curve was plotted. Figure 5 shows the lymphocyte pattern of all 55 persons. Their physical dose estimated ranged from 8-8800 cGy. It is obvious that not much information can be derived from this graph except to

conclude that there is, in most of the patients, an early lymphocyte drop after irradiation.

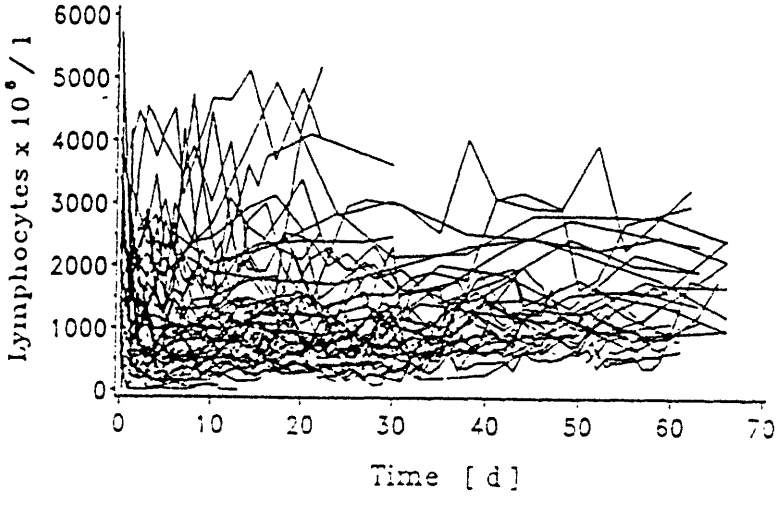


Figure 5

However, if one takes patient by patient and tries to bring the individual response patterns into response categories, it becomes obvious, that there are some 4 distinctly different patterns to be recognized.

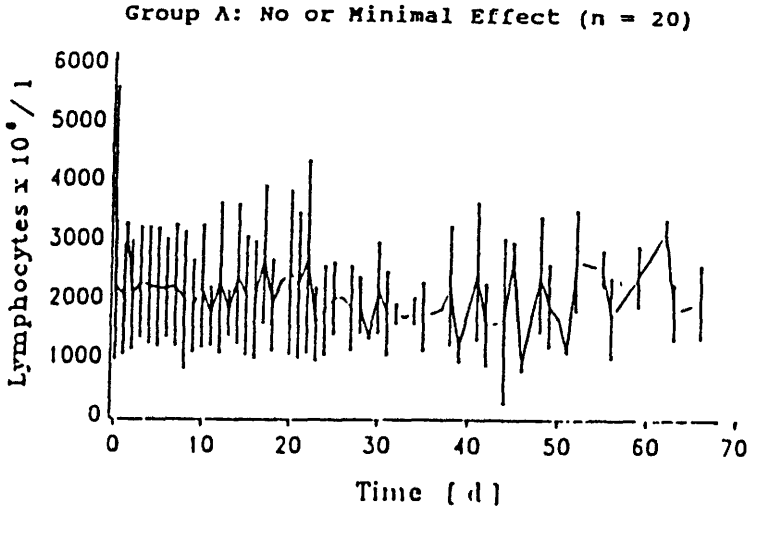


Figure 6

This category group A contains all patients who demonstrated a lymphocyte drop during the first 4 days but who did not reach values at or below 1000 per mm^3. This pattern corresponds to no or minimal effect. There was a second group of patients: category group B (figure 7).

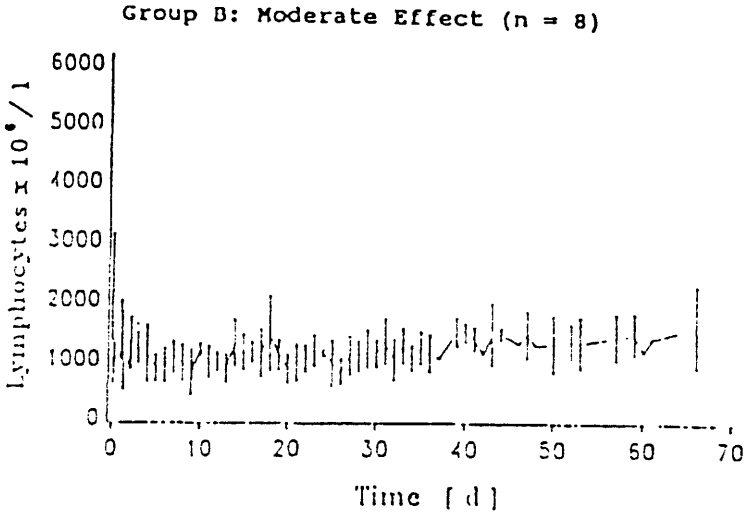


Figure 7

These patients showed an initial lymphocyte decline and the counts leveled off at the 800-1100 per mm^3 mark. It is interesting, that this group contains patients whose exposure dose was calculated to be between 120-240 cGy.

Figure 8 shows the pattern of lymphocyte changes who developed a severe course of the acute syndrome. There was an initial lymphocyte drop and a lymphocyte level of 200-800 per mm^3. There was a spontaneous recovery of hemopoiesis, including the lymphocytes.

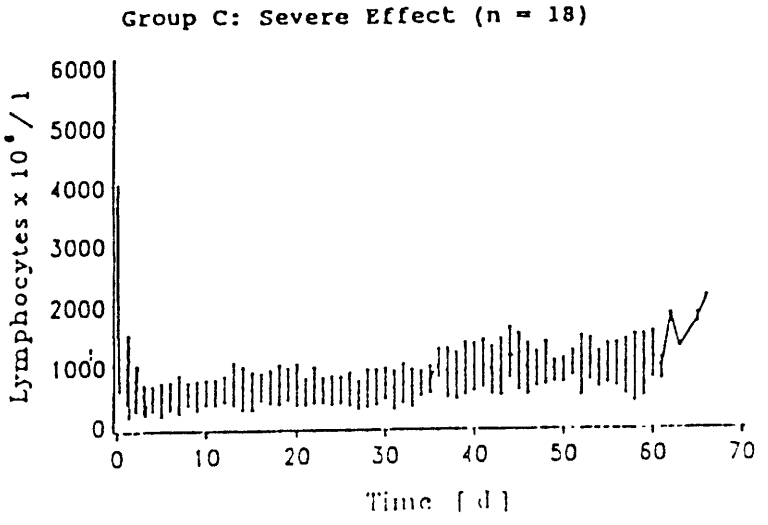


Figure 8

Figure 9 shows the lymphocyte pattern in the group of patients that developed a very severe form of the acute radiation syndrome. In all these patients there was a severe

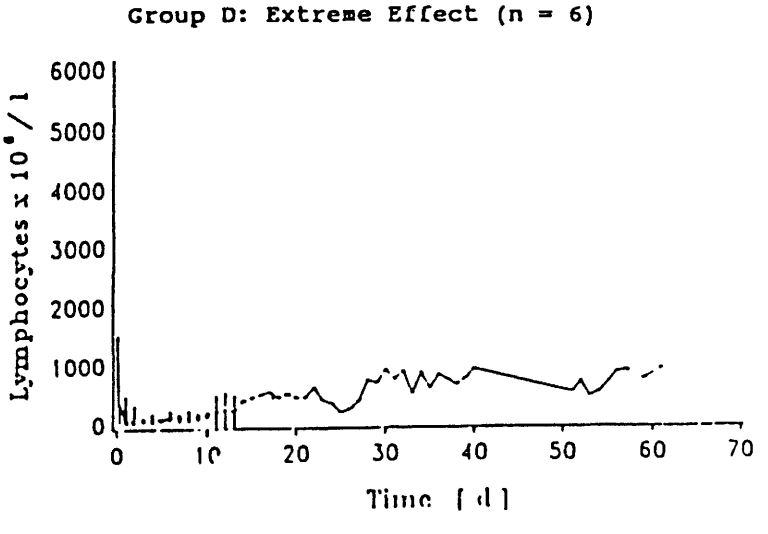


Figure 9

initial drop. The lymphocyte leveled off at below 200 per mm^3. This pattern is typical for an irreversible damage to the hemopoietic stem cell pool. The one patient who survived in this category was the recipient of a stem cell transfusion that was capable to restore hematopoiesis.

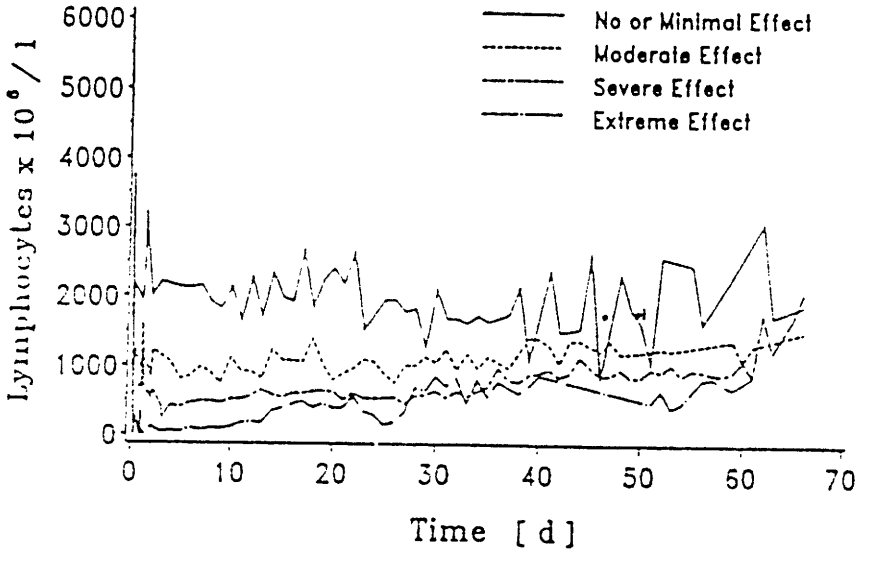


Figure 11

In summary (figure 11) one can relate the lymphocyte changes to the extend of damage to hematopoiesis. One may - at this point - confirm the notion, that there is no or little radiation damage if the lymphocyte count remains at levels above 1000/mm^3 within the first 2-4 days after exposure. One may also say, that there was a significant radiation exposure if the lymphocyte count declines to values below 1000/mm^3 within 4 days after exposure. However, it is difficult if not impossible to use the degree of lymphocyte depression within 4 days of exposure to predict whether hemopoiesis will recover spontaneously or not. If the lymphocyte evaluation, however, is combined with the evaluation of the granulocyte pattern, then one can state that a granulocyte decline to values of less than 200/mm^3

after 4-5 days and after an initial granulocytosis on days 1 and 2, combined with a severe lymphopenia as seen in group D of values below 200/mm³ is indicative of a degree of radiation injury that requires stem cell substitution if it is not a case that needs to be considered as a CNS-form of the acute radiation syndrome.

4. Pathophysiological models to evaluate the consequences of radiation exposure

From the analysis of previous accidents it is evident that the physician will not be able to obtain information on physical or chemical exposure (stress) estimates (measured in dose units). Such information would describe at best the amount and quality of external or internal stress experienced by the individual patient. Since the physician has to deal with an individual characterized by age, sex, genetic disposition and a previous personal health history, it must be the objective to analyse and determine the

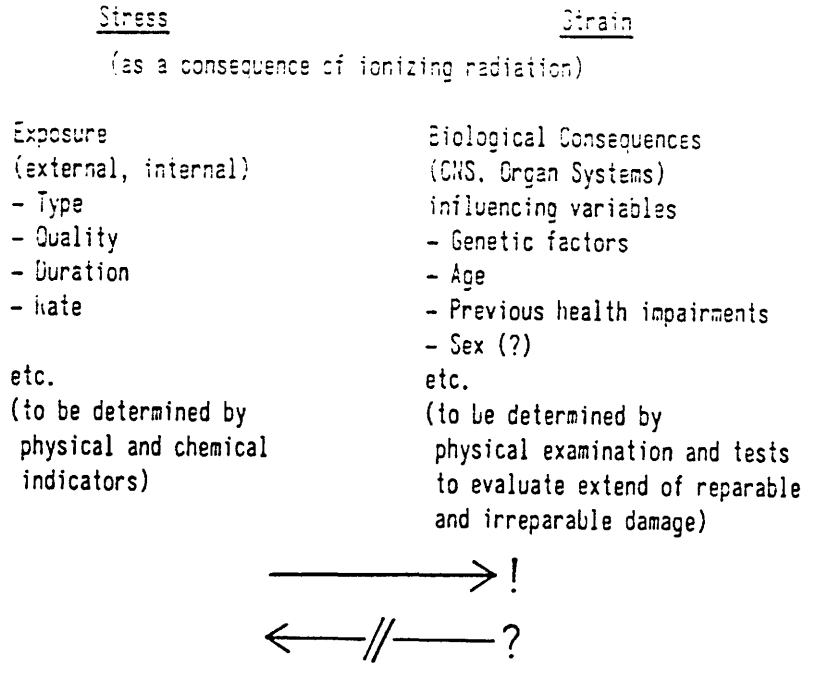


Figure 12

radiation induced strain. The physician has to rely on the analysis of biological indicators to determine the possibilities and limitations of a recovery of the organism as such and of the "critical organs" such as the skin, the central nervous system or, in other cases, the hematopoietic

system. Based on the experience of all accident casualties examined, it is essential to answer one single question: Is there a chance for a spontaneous recovery of the hematopoietic stem cell pool within a clinically reasonable period of time (i.e. some 2-3 weeks after exposure). If the answer is affirmative, then the therapeutic strategy will have to include the so-called "replacement" therapy (to replace temporarily missing functions such as that of granulocytes, platelets, red-cells, electrolytes etc.). If the answer is negative, indicating that a hematopoietic recovery is unlikely or would be too delayed to be useful, then "substitution" therapy needs to be attempted including transplantation of hematopoietic stem cells which may be available today from a variety of sources.

Information about the type, extend and quality of exposure which decribes the radiation induced "stress" for the individual is useful for medical management but is not decisive. It is not only unnecessary but sometimes misleading to use biological response patterns describing the "strain" experienced by a patient to extrapolate back to the "stress" that can be measured to determine "a dose" in air or in tissues.

The use of hematological indicators to predict the clinical course in a given patient after accidental exposure to ionizing radiation is of critical importance in certain phases of the management process. The "management decision tree" indicates that a number of questions need to be answered (Fig. 13). Initially, an answer is needed to the question: "Was the person exposed to ionizing radiation or not?" If the answer is affirmative, then new questions need to be answered, such as "Was there contamination with or without incorporation of radioactive nuclides" or "was there incorporation alone?" In all these instances, the additional question is: "Was there total or partial body exposure?"

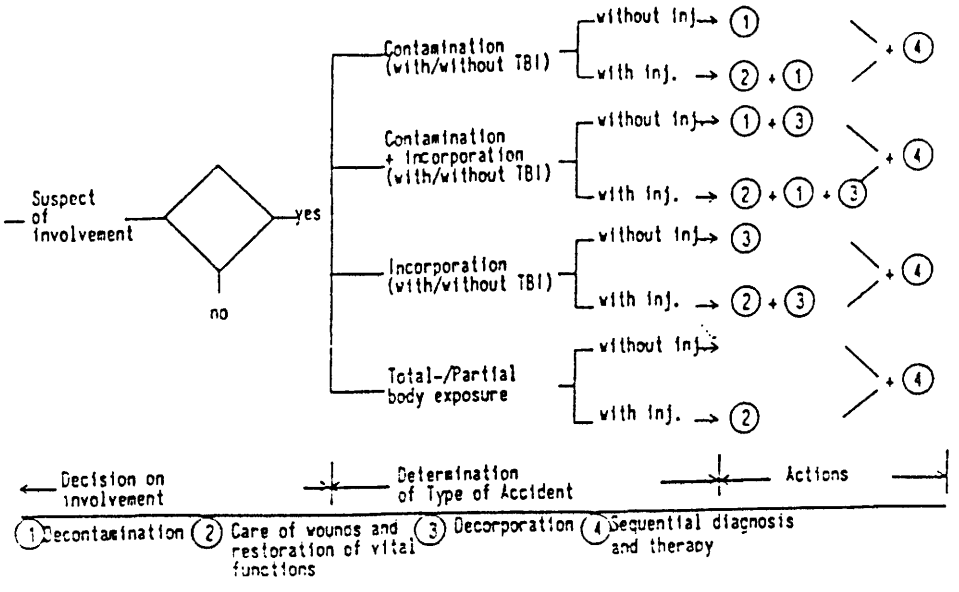


Figure 13

These questions will be relatively easy to answer using the information on hand and using simple instrumentation (monitors). However, it goes without saying that the answers to these questions will depend on anecdotal evidence and will only be qualitative. Exposure doses will not be available with any useful degree of accuracy. In the next phase, the medical personnel will have to determine the type and degree of additional physical, thermal or chemical injury present such as wounds or burns. The answers to these questions are very important in order to initiate appropriate therapy, such as management of external burns or surgical interventions in case of wounds. It becomes obvious that decontamination and care of wounds as well as restoration of vital functions take precedent over decontamination procedures. Finally, the "sequential diagnosis process" to determine whether or not a classical acutee radiation syndrome will develop, the type it will be and what type of clinical management should be used, will be possible.

What does "sequential diagnosis" mean or imply? The experiences gathered from post radiation accidents indicate that there is a typical sequence of biological events and changes that depend on the type and degree of damage inflicted upon hematopoietic and epithelial cell renewal systems. It is especially important to know the type and degree of damage caused in the stem cell pools. It is important to explore whether the damage inflicted on the stem cell pools is reversible or not. We have no direct measure of the stem cell pool damage in man. However, as far as hematopoiesis is concerned, the pattern of blood cell changes as a function of time after radiation exposure is so typical for the extend of stem cell pool damage that one is allowed to extrapolate back from the blood cell response pattern. Since this pool is distributed throughout the skeleton (reportedly consisting of more than 200 individual bones), any degree of non-homogenity in irradiation of the body due to the radiation pattern or due to the specific position of the body within the radiation field will affect the cell pool damage. Since it is not possible to extrapolate from <u>one</u> "blood cell count" back to the extend of stem cell pool damage, one has to determine the pattern of blood cell changes during the first 5-10 days after exposure in order to know whether one has to assume a reparable or irreparable damage to the hemopoietic stem cell pool.

From this logie it follows that one may have already gained relevant information about the probability of stem cell damage by day 1-2 after exposure by observing granulocytosis, lymphopenia and mitotically connected abnormalities in bone marrow smears (Fig. 3). However, by day 5, a definitive answer is possible regarding the question whether a spontaneous hemopoietic recovery can be expected or not (for pathophysiological mechanisms involved see ref. 1). This answer can be based on the pattern of

granulocyte, lymphocyte, platelet and reticulocyte changes during the first 5 days after radiation exposure. During that period, the medical personnel will try to assure the patients survival in case of severe injury to the CNS, to the cardiovascular systems or to the skin or injury inflicted by thermal burns or chemicals. "Sequential diagnosis" thus denotes the attempt to determine by daily thorough examination of the patient the development of changes of the hemopoietic and other cell renewal systems and the development of changes in other body systems (CNS, skin) with or without therapeutic interventions. In other words, for the management of individuals exposed to ionizing radiation it is useful to have quantitative information on the details of external and internal stress (i.e. dosimetry) but this is not essential. It is essential, however, to determine the resultant strain and to analyse by appropriate indicators such as the course of granulocyte and lymphocyte changes in the blood, the extend of reparable injury. This is necessary to plan and perform appropriate therapeutic measures.

In order to establish - at this point in time retrospectively - the radiation induced strain to the hematopoietic stem cell system as one of the key biological indicator to predict the clinical course of patients and to plan intervention strategies accordingly, a biomathematical model was developed to determine the number of stem cell units remaining from which a spontaneous regeneration might be expected to occur.

The basis for this computer model has been described elsewhere in detail (3). It has been adapted to simulate the granulocyte pattern observed in radiation accident victims. This computer model consists of a series of cellular and two regulation compartments (Fig. 8). It has been found to be useful in dogs and man for describing physiological and

pathological perturbations of the granulocytic cell renewal system such as a leukapheresis response, a response to cyclophosphamid administration, a response to total body irradiation with or without stem cell transplantation. In this model, to simulate granulocyte changes after total body exposure, it is important to indicate that the stem cells need to be categorized into 3 groups: 1. destroyed stem cells (no evidence for repair at all), 2. damaged stem cells with limited reparability and, 3. stem cells intact or with complete reparability. In this model (fig. 8) the stem cell pool containing the eliminated or destroyed stem cells ist not shown. The 2 compartments shown contain as "intact stem

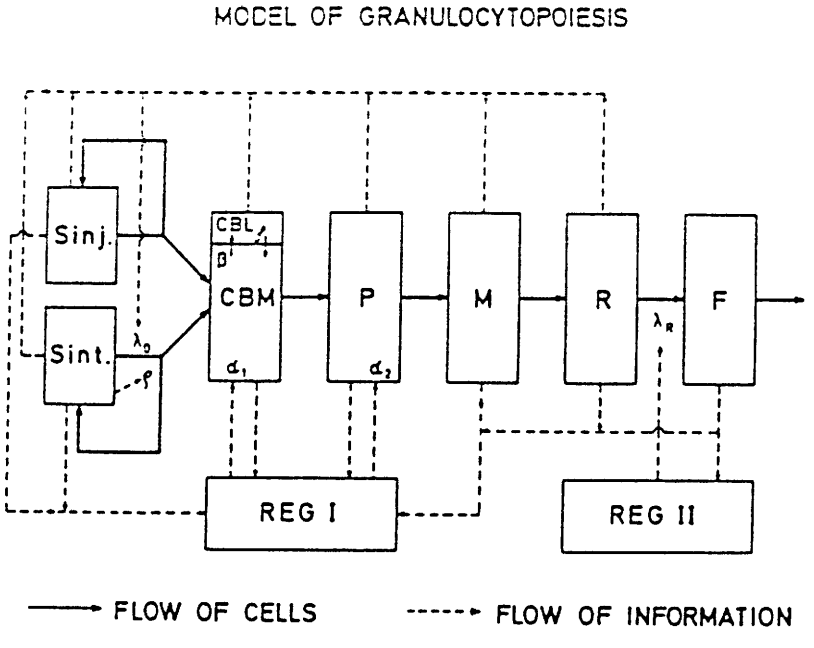


Figure 14

cells" those that were not irradiated at all (i.e. because they were localized during whole body exposure in a shielded part of the body) or are capable of complete reparation. The compartment "injured stem cells" coontain those the damage of which is partly reparable os that they are able to undergo one or several replicative cell divisions before

dying out.

For simulating the granulocyte changes after total body exposure it had to be assumed that the cellular contents of pools S, CBM and CBL as well as P can be reduced by radiation exposure in accordance with a D_o of 100 cGy. It was further necessary to assume that a certain fraction of stem cells already have or have regained a normal (essentially unlimited) replicative potential while another (usually larger) fraction is endowed with only a restricted replicative potential.

In order to try to calculate the number of stem cell units from the granulocyte changes in the blood stream observed during 60 days after accidental whole body exposure, 10 patients were selected out of the more than 300 so far incorporated into the Ulm radiation accident data base. All 10 patients had a typical form of an acute radiation syndrome and the course of clinical events was largely determined by the hematological responses. They were assigned to several "granulocyte response categories". Three patients (from the Oak Ridge accident 1958 and the Tennesse University accident 1975) were assigned to category II (figure 15).

Figure 15 shows a typical but "mild" course of events. It can be seen that there is an initial granulocytosis, then a phase of granulocyte decline between days 1 and 10. The granulocyte pattern between day 10 and 30 indicates continued granulocyte production and release of varying degrees. The granulocyte nadir is reached between day 30 and 40 followed by a slow recovery to stable normal values. The solid line is the result of simulating the course of granulocyte changes by assuming a certain number of reparable stem cells that remain in the stem cell pools from which the initial and the final system recovery occurs.

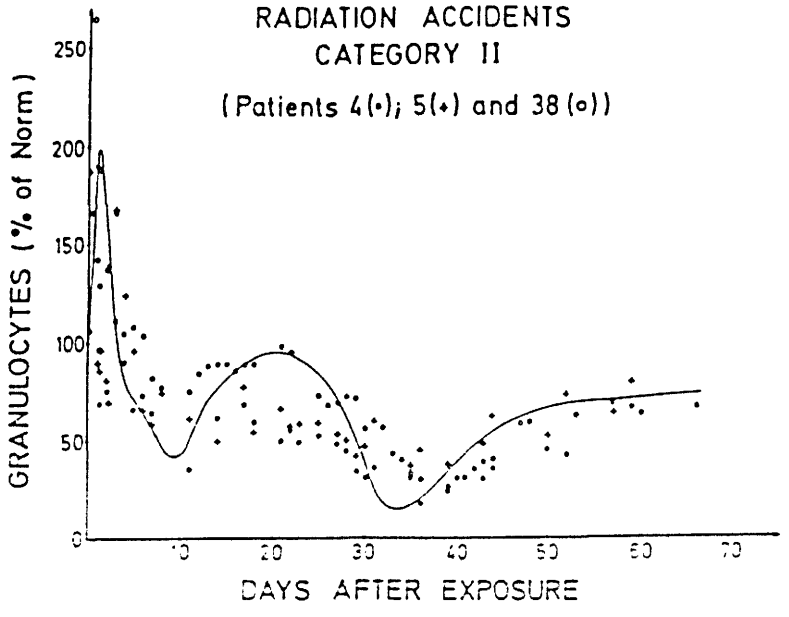


Figure 15

Figure 16 shows the granulocyte pattern of 2 patients involved in the Oak Ridge Accident of 1958. The pattern observed here is similar to that described for patients in

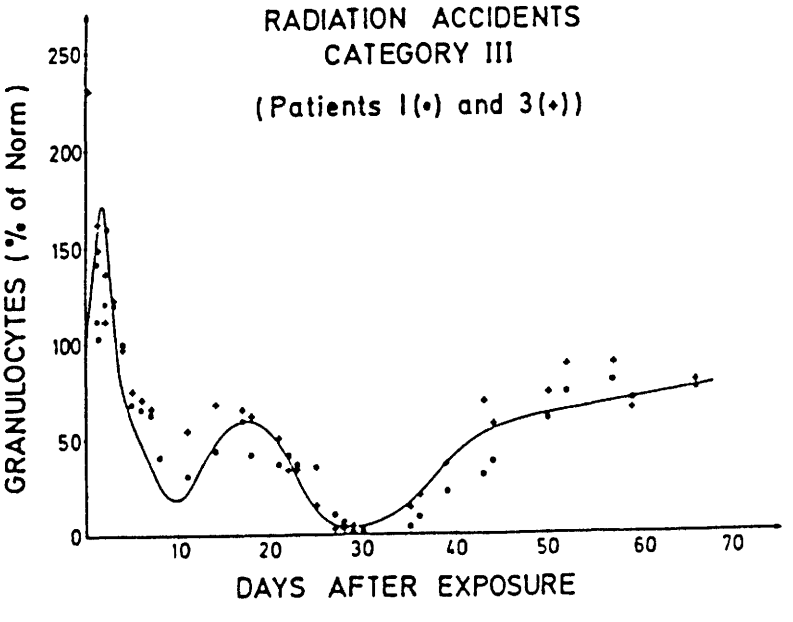


Figure 16

category II but more pronounced. There is a distinct initial granulocytosis (days 1-3), a primary granulocyte decline (days 3-10), an abortive rise (days 10-25), a secondary decline with a nadir around day 30 and a final recovery beyond day 35-40. The solid curve is the result of a computer simulation assuming a certain number of reparable stem cells in the stem cell pool (see below). The granulocyte changes are even more pronounced in the 3 patients assigned to category IV (from the Vinca accident 1958) (Fig. 17). This is a pattern very close to failure of hemopoietic recovery but attainable with intensive

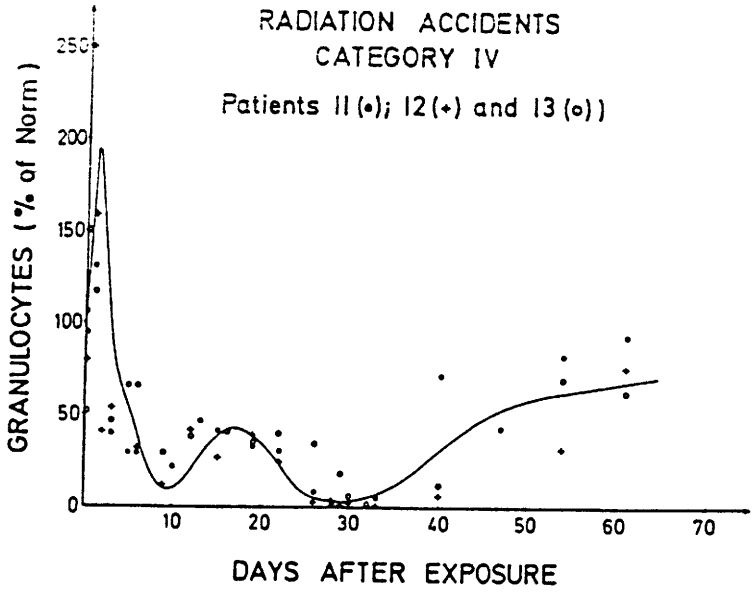


Figure 17

replacement therapy. The primary granulocyte decline during the first 10 days is extensive. But it is essential to realize that between days 5-10, there are day by day measurable levels of granulocytes in the peripheral blood (above 200-500 per mm^3). There is evidence for an abortive

rise between days 10 and 20 and a secondary decline and final nadir around days 25-35. There is, however, a spontaneous recovery beyond day 35. The computer curves simulate this pattern and are based on a certain number of reparable stem cells which are present in the stem cell pools. There are so far no observations between this category IV and the patients of category V characteristic of a response pattern after an irreparable damage to the stem cell pool. The granulocyte response category V contains 2 patients (from the accidentss in Los Alamos 1946 and Brescia 1975) (Fig. 18). There was an initial pronounced granulocytosis during days 1-3 or 4 and an abrupt decline of

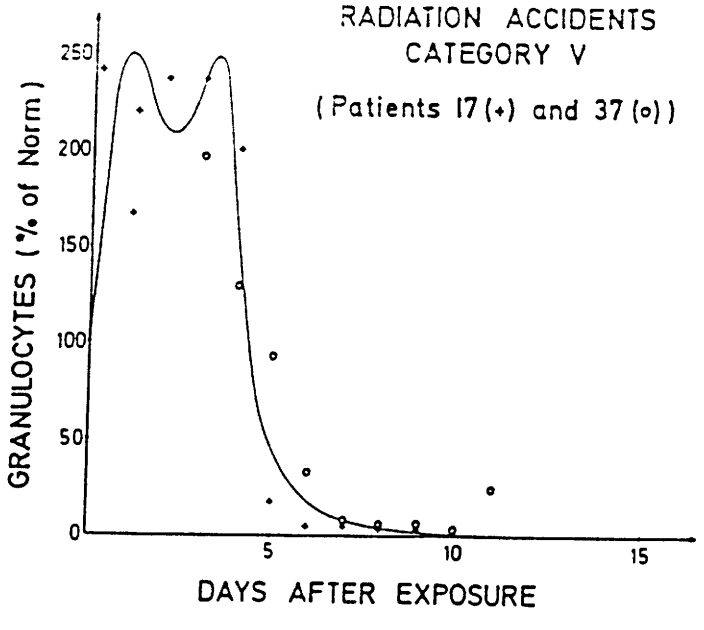


Figure 18

blood granulocyte concentrations between days 4-6. This is indicative of a complete block in granulocytopoiesis at the myelocyte-promyelocyte and stem cell level (granulocyte values below 200 per mm^3).

There is a severe granulocytopenia combined with a significant thrombocytopenia and lymphopenia present on day 10. The clinician would describe the findings on day 10 as "severe aplastic anemia" without a chance of spontaneous recovery. The computer derived curve simulates the granulocte pattern using certain numerical assumptions as discussed below.

It is of interest to use this computer model of granulocytopoiesis to calculate how many stem cell units of different quality of limited or unlimited replicative potential must have remained in the four granulocyte response categories shown to allow for regeneration or failure. In table 5 it can be seen that the number of destroyed or irraparably damaged stem cell units increases as the granulocyte response pattern "gets worse". This means that the granulocyte pattern becomes more distinct in relation to the initial granulocytosis, the primary granulocyte decline, the extend and duration of abortive rises, the time and duration of secondary decline and the granulocyte nadir and course of final recovery.

Computer derived Calculations of Stem Cell Pool Sizes

Radiation Response Category	Remaining Intact Stem Cells % (Cell number)	Remaining Injured Stem Cells % (Cell number)	Destroyed Stem Cells %	Ranges of Physical Dose Estimates in cGy
Category II Pat. 4,5,38	0.008 (1.0×10^5)	24.0 (3.0×10^8)	76	127 - 236
Category III Pat. 1, 3	0.0024 (3.0×10^4)	4.8 (6.0×10^7)	95	339 - 365
Category IV Pat. 11,12,13	0.0008 (1.0×10^4)	2.4 (3.0×10^7)	97.6	323 - 426
Category V Pat. 17, 37	0 (0)	0.0006 (7.7×10^3)	99.9994	1.114 - 1.200

Table 5

But it is of great interest that in the categories II, III and IV, the granulocyte response pattern is compatible with the notion that there are stem cell units remaining after radiation injury. Some are intact or are completely reparable and some are of restricted reparability. It appears as if there is a clinical "threshold" for the reversibility of damage to the granulocytic cell renewal system. There must be at least 0.0008% or 8 in 10.000 stem cells intact, or completely repaired to allow a spontaneous recovery. There must have been between 2.4 and 24.0% of stem cells with restricted reparability, responsible for the appearance of an abortive rise.

It is obvious, that this type of granulocyte response categorization allows one to determine the radiation induced strain in a given patient (see Fig. 1). It may well be that this strain may be influenced by individual parameters such as previous health impairments genetic factors, age or sex. Such an analysis shows the potential in allowing the quantification of an individual strain in terms of remaining stem cells which are intact or repaired completely from which the final recovery can commence.

5. Hematological indicators to predict the course and outcome of acute radiation syndromes

The Ulm radiation accident data base and the biomathematical simulation model of radiation injured hematopoietic cell renewal systems will have to be completed and extended to evaluate more patient data and to include more cell systems, such as megakaryocytopoiesis, erythropoiesis. This information will eventually be used to develop a knowledge based expert system to be useful in the management of radiation injured persons. This development, however, will require further intensive work in the Ulm Radiation Medicine Research Group.

However, on the basis of available knowledge and experience it appears possible already today, to describe constellations of blood cell changes early after accidental whole body irradiation that are very useful in predicting the most likely clinical course of an irraidated patient and to plan the essential therapeutic strategies.

The key question to be asked in the "sequential diagnostic process" subsequent to an accidental total body exposure is: "Can one expect a spontaneous hematopoietic recovery or not?" At this moment, there is good probability for an answer within 6-7 days after an accidental exposure to ionizing irradiation. but it is possible to predict the course of events even earlier, that is 3-5 days after an accident. Such predictions will always have to be based on very carefully performed evaluations and accurately recorded blood cell counts during the first 10 days. Aside from the granulocyte count, one may take into consideration also the concentration of lymphocytes, platelets, reticulocytes and red cells.

The blood cell response pattern typical for irreversible damage to the hematopoietic stem cell pool is the result of the pathphysiology of the different blood cell renewal systems (cellular kinetics, radiobiological properties, functional and regulatory mechanisms) and can be described as follows.

If one finds on day 1 and 2 (24-48 hours after removal of the individual from the radiation field) a significant granulocytosis and at the same time a severe lymphocytopenia (less than 30-50% of pre-irradiation values) one can assume significant damage to the hematopoietic systems. This notion will be supported by examining bone marrow smears prepared from the most intense irradiation site. One would expect a large number of injured cells (nuclear edema, phagocytosis of cell nuclei but in particular mitotically connected abnormalities in normoblasts and other red cell precursors to some extend in all cell types capable of cellular proliferation) (2).

A high probability of an irreversible damage to the hemopoietic stem cell pool can be attached to the following observation to be made on day 5 after exposure: if on this day the granulocytes have declined or are declining to very low values (less than 200 per mm^3 on day 5 or 6) and if the lymphocyte concentration is also very low (less than 10-30% of normal) and if the platelets show a progressive fall with values on this day of some 40-60% of normal, then a spontaneous recovery of hemopoisis within reasonable periods of time is unlikely (see category V, figure 18).

It is possible to predict whether or not reversible or irreversible damage to hematopoiesis can be expected on day 10. If on this day the granulocytes are still below 300 per mm^3, the lymphocytes still low or absent and the platelets

approaching very low levels (less than 50.000/mm³), then the hemopoietic tissue must be injured to such an extend that a spontaneous recovery is unlikely.

In reverse, if the early pattern of blood cell changes is similar to the patients described in category IV (see figure 17) (some granulocyte and platelet production continuously present on the basis of cell kinetic considerations) then it can be assumed that the stem cell pool has a fair chance to recover and that the final hemopoietic recovery will become apparent (depending on the extend of stem cell pool damage) between days 30-40 after exposure.

These above considerations have important therapeutic implications. All patients described in categories II, III and IV experienced a spontaneous hematopoietic reecovery. Their therapy, however, had to take care of the transient electrolyte imbalance (due to damage to the intestinal surfaces: "radiation induced burns of the inner surfaces of the body"), the transient granulocytopenia (with infections complications requiring antibiotics or even granulocyte transfusions) and the transient thrombocytopenia resulting in bleeding episodes requiring platelet transfusions. In all these instances (supported in many instances by strict isolation or even bacterial decontamination to sterilize the g..t and the skin flora) hemopoiesis recovered spontaneously. It is likely, that these patients would have been able to recover faster if they had had an identical twin to serve as a stem cell donor or if there would have been a bank of cryopreserved autologous hemopoietic stem cells (from blood or from bone marrow).

In patients with a blood cell response pattern dentical or similar to that described for category V, there is only one possible curative therapeutic approach. These patients should be treated as if they were patients with "severe

aplastic anemia". For these patients one would look for a suitable stem cell donor (allogeneic bone marrow or blood stem cells). Since in many, if not all instances, one would not be able to judge the degree and sufficiency of immunological suppression by the radiation exposure, one would have to "condition" the patient by appropriate means (chemotherapy as in the preparation for stem cell transplantation for severe aplastic anemia).

6. Possibilities and limitations to treat persons suffering from acute radiation syndromes

The work performed within this EC-contract did not cover all aspects to treat radiation injured persons. The Chernobyl experience added a new dimension to the management of radiation injured persons, skin burns in addition to the total body exposure by penetrating ionizing radiation. In this report emphasis will be on the question of establishing a blood stem cell bank as a means to treat persons at high risk of being exposed as a consequence of radiation accidents.

In the previous chapter 4 it was pointed out that one must consider in principle 2 situations: radiation exposure categories resulting in a reversible hematopoietic damage and those resulting in an irreversible hematopoietic damage.

The Chernobyl accident (see tables 1 and 2) providul evidence that all persons in Grades I through III need to be considered as having been exposed to such an extend that the hemopoietic damage was reversible. The fatal casualties observed in grad III are attributable to the severe burns of the skin.

Aside from considering the therapeutic strategies with respect to skin burns and incorporation of radioactive nuklides, the therapy of choice for taking care of the consequences of the transient hemcpoietic failure, in particular infection and bleeding has been described as replacement therapy (4). It consists essentially of trying to remove exogenous and endogenous bacteria and other microbial organisms from the skin and the gastrointetinal tract by antibiotic therapy. It is in cases of severe granulocytopenia advisable to induce a "gnotobiotic state" and to treat the patient in a sterile isolation system

(reverse isolation). In this way, one can wait for the granulocytes to recover on or around day 30 after total body irradiation and one can also tolerate low platelet counts without high risk of bleeding. This form of therapy has been described previously.

If there is a severe thrombocytopenia with a risk of bleeding, it may be necessary to perform platelet replacement therapy as was done in one of the Chernobyl cases (see figure 2).

It may also be necessary to take special care of the electrolyte balance, especially if some form of gastrintestinal damage becomes obvious (diarrhea).

In this report, sufficient evidence was given for the fact, that there is, after acute radiation exposure, the onset of spontaneous granulocyte and platelet recovery on or around day 30. It is thus necessary to provide optimal care during this transient phase of hematopoietic failure (gnotobiotic care, provision of adequate fluids, platelet support if necessary).

What about stem cell substitution? If there is evidence of an irreversible damage to the stem cell system as described above, it is essential to provide hematopoietic stem cells for restoring the hematopoietic function of the bone marrow. Other research institutes participating in this Post-Chernobyl programme have concentrated their efforts on the improvement of bone marrow transplantation.

The Ulm Research Group together with the Group of Professor Hunstein in the Medical Department of the University of Heidelberg has established the concept of a <u>blood stem cell bank</u>. This approach may become useful if there are persons at high risk for radiation exposure who are prepared to

allow their pluripotent hematopoietic stem cells to be collected from the peripheral blood, stored at ultralow temperatures and being antotransfused if necessary after unexpectedly high radiation exposure. This risk may become evident after a radiation accident when the necessity arises to engage in clean-up operations. In the Chernobyl situation, there was evidence for radiation exposure in the teams of technicians participating in rescue and clean-up operations.

The blood stem cell bank concept has been developed at the preclinical level (dog model) at the University of Ulm and at the clinical level at the University of Heidelberg.

The concept of an autologous blood stem cell bank was developed as a means to treat patients with acute myelogenous leukemia (AML). All transplants were performed in the first remission following conventional induction chemotherapy.

The patients data are listed in table 6.

		ABSCT	ABMT	p value
no patients		20	23	
male		12	14	n.s.
female		8	9	
age (median, range)		41 (5-48)	33 (17-50)	p = 0.05
WBC at diagnosis (median, range)		5300/ul (500-268,000)	17600/ul (700-277,000)	n.s.
time between start of CR preceding transplant, and transplant (median, range)		3,5 months (2-12)	4 months (2-6)	n.s.
FAB	M1	4	5	
	M2	5	2	
	M3	2	3	n.s.
	M4	7	9	
	M5	2	4	

Table 6

Blood stem cell collection

Blood derived hemopoietic stem cells were collected by continuous flow cytopheresis using a Fenwal CS 3000 blood cell separator (BAxter Deutschland GmbH, München, Federal Republic of Germany). For mobilizing stem cells from extravascular sites into the circulation, aphereses were performed at the time of bone marrow recovery following chemotherapy-induced transient myelosuppression. The regimen used was identical to the consolidation treatment of AML: ARA-C 100 mg/m² q 12 hr s.c., days 1-5; Daunorubicin 45 mg/m², days 3 and 4 (11). Stem cell aphereses were initiated when the patient had reached a peripheral WBC count of 1,000/ul, and a peripheral platelet count of 40,000/ul. The median number of aphereses per patient was 10 (range, 6-14); the time interval between aphereses was 1-3 days.

The MNC-enriched peripheral leukocytes were collected according to a program designed by the Baxter Company (12). The median percentage of MNC collected from 199 aphereses was 91,7% (range, 50.8-100).

The harvested MNC-enriched leukocyte suspension was then pelleted and resuspended with ABO compatible human plasma to a final volume of 50 ml, and transferred to a freezing bag (Delmed, Canton, Mass.). 50 ml of cold S-MEM supplementted with 20% DMSO were added to end up with a final DMSO concentration of 10%.

Marrow collection and processing

The technical aspect of bone marrow collection and processing has been described in detail elsewhere (10). Briefly: marrow was collected by multiple aspiration from the posterior iliac crest and the sternum. The cell suspension was depleted of red blood cells (RBC) and most of

the polymorphonuclear cells (PMN) by a two-step procedure: a buffy-coat and a Ficoll-Metrizoat (FM) gradient centrifugation using the IBM Blood Cell Processor 2991 as described by Gilmore et al. (13).

Mafosfamide(R), a stable substitute of the activated primary metabolite of CY, was added at a concentration of 60 to 80 ug per $2x10^7$ WBC and was incubated with agitation at 37°C for 30 min. Cells were washed, resuspended with ABO compatible human plasma and transferred to freezing bags, 50 ml per bag. 50 ml of cold S-MEM supplemented with 20% DMSO were added to each bag.

Blood stem cell / marrow cryopreservation and transfusion

The cell suspension was then immediately frozen at a controlled rate of 1-2°C/min from +10°C to -70°C/min up to -100°C (BV-10 Biological Freezer, Cryoson GmbH, Federal Republic of Germany). The frozen bags were stored in liquid nitrogen. Thawing was performed rapidly in a 42°C waterbath without the DMSO being removed. The cell suspension was then aspirated into 50 ml syringes and immediately injected into the patient's central line (Hickman catheter) at a rate of 10 ml/min.

The median total volume of cell suspension transfused per patient was 1025 ml for ABSCT patients (range, 600-1520), and 400 ml for all ABMT patients.

Pretransplant regimen

The hyperfractionated TBI was slightly modified according to Shank et al. (14). The median total body dose at body center was calculated to be 14.5 Gy (range, 14.4-15.3) for ABSCT patients, and 14.4 Gy (range, 12.1-16.7) for ABMT patients. 120 cGy per fraction were given 3 times daily on days 1, 2,

3, and twice on day 4, four hours apart for 11 fractions (total 12.1-13.7 Gy) or 12 fractions (total 13.8-14.9 Gy), three fractions per day. The dose range between 15.0 and 16.7 Gy included 12 fractions, 130 cGy each. A 23 MeV linear accelerator source (Saturne) (38 cases) or, alternatively, a Co^{60} gamma source (3 cases) was used with a mid-line dose rate of 7-18 cGy per min. The lung dose was limited to 9 Gy. Additional boosts with electrons of a maximum of 15 MeV (depending on the chest wall thickness) were given to the previously shielded areas, particularly the ribs, to reach the total maximum dose. Following TBI (days -9, -8, -7, -6) CY was given i.v. on each of 4 consecutive days (-5, -4, -3, -2) at a daily dose of 50 mg/kg. After one day of rest transplantation was performed on day 0.

Two more patients were treated with busulfan (16 mg/kg) and CY (200 mg/kg) prior to blood stem cell (1 case) or bone marrow transplantation (1 case).

Intensive care post transplantation

The patients were kept in reverse isolation from the beginning of high dose CY until a peripheral PMN cell concentration of 500 per ul was reached. Prophylactically they all received acyclovir, ketoconazole and CMV-hyperimmunoglobulin together with partial antibiotic decontamination of the gut (15) up to 100 days post transplantation. When fever exceeded 38.5°C broad spectrum antibiotics were administered, and, when fever persisted for more than 4 days despite appropriate antibacterial treatment amphothericin B was added systemically. All platelet support was HLA-A/B matched and CMV negative if the patient was negative as well. All blood products were irradiated with 20 Gy to avoid the risk of GvHD induction.

Statistical evaluation

Clinical and laboratory data were obtained from the bone marrow transplant data base and analysed by standard statistical methods using the SAS software program. Basic characteristics of the patient groups were compared by means of chi-square tests. In the case of uncensored quantitative variables the usual Mann-Whitney-Wilcoxon-Tet was applied. Plotting the distribution of censored variables and estimating median values was carried out according to the Kaplan-Meier method. The main variable of interest was DFS, defined as the time from transplantation to relapse. Three patients died without relapse, two of them were counted as "events" and one was considered as a censored observation, because of death due to a generalized Herpes virus infection. The comparison of ABMT and ABSCT or prognostic subgroups with respect to DFS was based on the log rank test. Theh estimation of a "cure rate" did not seem appropriate, instead we calculated rates for two-year disease-free survival with confidence intervals based on the standard errors supplied by the Kaplan-Meier method. Other censored variables such as hemopoietic reconstitution and duration of hospital stay were analysed in the same way, but tests were based ont eh generalized Wilcoxon statistics. Formal significance levels of $p = 0.05$ were used without adjustment for multiple testing.

The probability of DFS or relapse was calculated from the day of transplantation (day 0) and was analysed as of June 1, 1989.

Harvesting and transfusion of cells

To guarantee a sufficient blood stem cell autograft, its total number of pluripotent stem cells should be comparable with that from the marrow. Since blood stem cell

concentration is only about 1/10 to 1/20 of that in the marrow, the total number of blood MNC harvested had to be adjusted.

The median yield of MNC collected by a single continuous-flow apheresis was 5.4 x 10^9 (range, 0.2-14.5) or 13 x 10^4 CFU-GM (range, 1-180). The total number of blood derived MNC transfused per kg b.w. was 80.0 x 10^7 (range, 17.3-140.9) or 2.35 x 10^4 CFU-GM (range, 0.2-4.1). The marrow stem cell graft, on the other hand, contained 4.7 x 10^7 (range, 0.6-13.0) MNC at a median. The respective median number of marrow CFU-GM transfused per kg b.w. was low with 0.14 x 10^4 (range, 0.006-1.0) due to a selective loss of CFU-GM as a consequence of Mafosfamide(R) purging (15).

Hemopoietic reconstitution

The total number of pluripotent stem cells and WBC as well as cell composition of the autograft determines the characteristic kinetics of hemopoietic reconstitution following myeloablation and ABSCT or ABMT. As Figure 19 shows on a daily blood count basis, the reconstitution of peripheral leukocytes started significantly earlier following ABSCT compared to ABMT. The difference in speed of hemopoietic reconstitution of peripheral leukocytes started significantly earlier following ABSCT compared to ABMT. The difference in speed of hemopoietic reconstitution within 28 days after transplantation was highly significant each day from day 1 on ($p = 0.0001$). The median time it took for the peripheral WBC count to reach 1000/ul was 10 days (ABSCT) compared to 28 days (ABMT) ($p = 0.0001$) (Figure 2) or 14 days (ABSCT) compared to 42 days (ABMT) ($p = 0.0001$), which was the median time it took for the peripheral PMN count to reach 500/ul. The kinetic of platelet reconstitution were in favour of ABSCT patients; the median time to reach a stable 20,000/ul platelet concentration was 30 days (ABSCT)

compared to 46 days (ABMT) with a borderline significance (p = 0.05). Three out of 20 ABSCT patients did not reach a stable 20,000/ul platelet count before day 100 post transplant compared to 8 out of 22 evaluable ABMT patients.

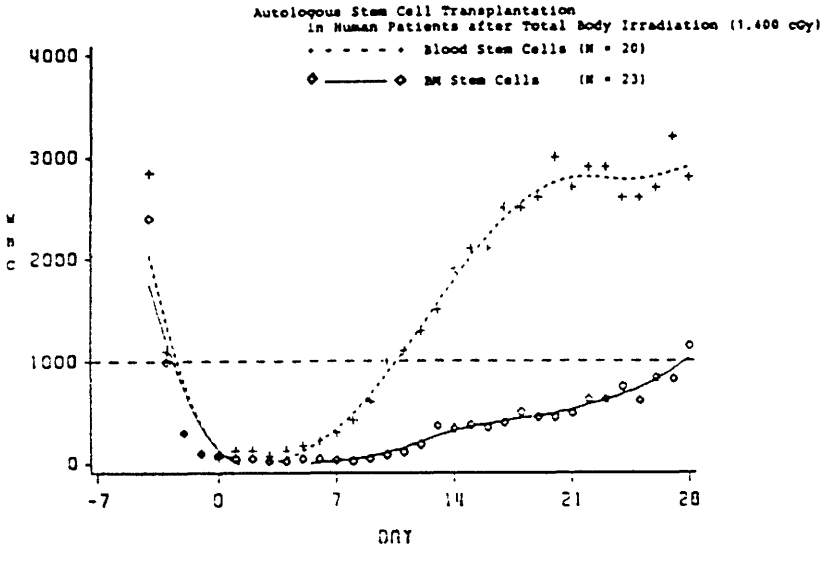


Figure 19

Early transplant-related morbidity and mortality

Transient supraventricular tachycardia and severe mucositis were seen in almost all patients. One ABMT patient died of septicemia, whereas there was no mortality among the ABSCT patients. The median hospital stay was 45 days for ABSCT patients versus 73 days for ABMT patients (p = 0.005).

Due to the rather large volume of the blood stem cell autograft (1025 ml at a median) transfused over a central line, patients suffered from chills, stomach ache and muscle cramps at the lower extremities. Those side effects ceased shortly after transfusion was concluded, and were not as

severe during marrow cell transfusion, when an autotransplant at a lesser volume (400 ml) was carried out.

Disease-free survival

When compared statistically, there were no differences to be observed in both the ABSCT or ABMT groups as far as sex, WBC concentration at diagnosis, the time between start of remission and transplant, and FAB subclassification were concerned, while the median age of ABMT patients was less than that of ABSCT patients ($p = 0.05$) (Table 1).

The median relapse time for the 20 ABSCT patients was 14.3 months, and 37% were disease-free two years after transplantation (95% confidence interval from 13% to 60%). In the group fo 23 ABMT patients the two-year DFS rate was 59% (95% confidence interval from 38% to 80%), to date no relapses have occured later than 9 months post transplant. As can be seen in Figure 5 the Kaplan-Meier estimates for DFS in both groups are crossing and the long rank test is not significant ($p = 0.33$). Of 20 ABSCT patients autografted in 1st CR, 11 have relapsed; of 23 patients autografted with bone marrow, 9 have relapsed or died too early to be evaluated (1 patient).

Relevance for radiation accident management

It is apparent from these clinical data, that it is feasible to set up blood stem cell banks in order to collect and store pluripotent hematopoietic stem cells from the peripheral blood of persons who are at high risk to be exposed in extraordinary circumstances, for instance in radiation accident clean-up operations. Further work, however, is essential in this field in two aspects. It appears of interest to develop new approaches to mobilize stem cells into the blood from extramedullary sites. In

patients, the high yield of blood stem cells was obtained by pretreatment with cytotoxic drugs, such as ARA-C and Dannorubicin. Cyclophosphamide has also be shown in dogs and man to produce a high concentration of hematopoietic progenitor cells in the blood within 10-14 days. In dogs, it has been shown that dextran sulfate (DS) is capable of inducing a significant increase of ciruclating progenitor cells within 3-4 hours after DS administration. It appears therefore useful to explore the mechanisms for this type of mobilization and to transfer the experience gained to the clinical level. A second area requiring further intensive research is evident if one considers the advances made in utilizing recombinant hemopoietic growth factors in the treatment of hemopoietic failure. The question is whether one could utilize very smal numbers of blood derived stem cells and enhance their replication and differentiation potential by post-transfusion application of such recombinant growth factors. In this case, one might be able to collect a sufficient number of pluripotent blood derived stem cells with one single leukapheresis.

7. Further research needs to improve existing and develop further strategies to handle persons accidentally exposed to ionizing radiation

It is apparent from this report that a lot of progress has been made in trying to improve means and ways to adequately take case of persons accidentally exposed to ionizing radiation. However, a lot of mon work has to be done which may be summarized as follows.

The Chernobyl experience should be fully analysed by establishing appropriate collaboration projects with the scientific community of the USSR. It is reported, for instance, that there were many more people exposed to ionizing radiation the clinical data of whom have not been adequately analysed. There are also a large number of persons who participated in clean-up operations who may have had significant radiation exposures.

The goal of such a collaboration would be to set up an internationally accessible data base of persons accidentally exposed to ionizing radiation possibly in a continuous or protracted way. This type of research would be of great importance for radiation protection planing in Europe.

There is a great need to analyse all clinical data that become available in the future of accidentally radiation exposed persons. There are - most likely - a relatively large number of persons exposed to high dose of radiation that have not as yet been published in the international literature. It is desivable to establish an international data base for a many cases of acute accidental radiation exposure as appropriate.

On this basis, it would become of importance to develop an knowledge based expert system for assisting medical doctors

in the decision making in case of radiation accidents. This would require a further analysis of the pathophysiological mechanisms of radiation induced changes in cell renewal systems at risk employing also biomathematical computer simulation technics.

Of particular importance is the development of further biological markers for radiation exposure. Blood cell changes need to be complemented by more sophisticated methods mensing damage and complete or incomplete repair at the subcellular and molecular level. This approach will become essential especially if the problem of medium to long term radiation exposure is to be considered.

There is also need for further research to improve existing and develop new approaches to the treatment of radiation induced health impair ments. The advent of recombinant factors such as IL-3 alone or in combination with erythropoitin signals new possibilities to influence hematopoietic recovery positively. Therefore, it is mandatory to do more research in the field of using such recombinant factors in cases of radiation induced hemopoietic failure.

Finally, it appears essential to set up a European network of radiation medicine centres to establish a collaborative system for handling radiation accident cases if necessary. This network should also take over the responsibility to train a sufficient number of European physicians to participate - if necessary - in the optimal can of radiation accident victims.

INSTITUT DE PROTECTION ET DE SURETE NUCLEAIRE

DEPT. DE PROTECTION SANITAIRE

SERVICE D'HYGIENE RADIOLOGIQUE

CONTRACT BI6 - 0285 - F

STUDIES OF SIDE AND LONG TERM EFFECTS OF HEMATOPOIETIC GROWTH FACTOR THERAPIES

J. C. NENOT.

STUDIES OF SIDE AND LONG TERM EFFECTS OF HEMATOPOIETIC GROWTH FACTOR THERAPIES

1 GENERAL

Hematopoiesis is under the control of growth and differentiation factors which allow the organism to adapt itself to the new situations by consequently modulating its own response. Some of these factors are well known, some others are only hypothetical. The most studied factors are:

-the Granulocyte-macrophage colony stimulating factor (GM CSF)

-the Granulocyte colony stimulating factor (G CSF)

-the growth factor for the red cell line: Erythropoietin (EPO)

-the growth factor for undifferentiated cells: interleukin 3 (IL 3).

Those factors are small proteins which bind to specific receptors on the membrane of the target cells. They stimulate the proliferation or differentiation of hematopoietic progenitors. Furthermore, they act on the functions of mature cells (for exemple: the phagocytic function of granulocytes for G CSF).

Recent advances in the study and large scale production of these hematopoietic growth factors have allowed their use for therapeutic purposes. The main field of clinical investigations are:

- bone marrow toxicity control for antitumoral treatment (such as chemotherapy)
-treatment of global or elective aplastic anemia
-treatment of infection related leukopenia.

GM CSF is a protein acting at very low doses (picomolar concentrations) for the in vitro stimulation of colonies of differentiated granulocytes and monocytes (GM CFU: Granulocyte Macrophage colony-forming unit). The successful cloning and expression of cDNA encoding human GM CSF gene, has allowed the production of recombinant human (rHu) GM CSF which is now submitted to laboratory studies and clinical trials for few years. The recombinant human GM CSF bind to high affinity receptors on the membrane of the responding target cells and possesses biologic activity for the proliferation and/or differentiation of granulocyte, mixed granulocyte-macrophage, pure eosinophilic (CFU eo) and mixed cell colonies (CFU mix) from human normal blood, bone marrow and some leukemia cell lines (such as HL 60, where GM CSF acts as a weak inducer of differentiation, increasing the number of cells displaying monocytic or eosinophile differentiation patterns).

In the presence of erythropoietin, GM CSF stimulates also the growth of human erythroid burst forming unit (BFU E). GM CSF has a wide range of effects: in vitro and in animal studies it enhances

the functions of mature granulocytes, monocytes/macrophages and eosinophils on which its receptors are also present in small quantities. In functionnal studies GM CSF stimulates neutrophil viability, inhibit neutrophil migration (in vivo, in about 30% of the patients cellular migration into a sterile skin window appears to be reduced after GM CSF treatment), and stimulates superoxyde ion production in response to bacterial chemo-attractant.
Furthermore, GM CSF stimulates the neutrophil oxydative metabolism involved in granulocyte microbicidal or tumoricidal activities, so that the granulocytes appear to be activated and are more efficient in bacteria killing after GM CSF stimulation. The phagocytosis of bacteria and cytotoxicity are also stimulated by GM CSF.
GM CSF enhances the viability of eosinophils and act on the antibody dependent cell-mediated cytotoxicity (ADCC) and on antitumoral activities of macrophages. Furthermore when associated with other growth factors it helps to the development of red cell progenitors and megakaryocytes (thus acting on platelet renewal). The GM CSF has been used in a variety of clinical settings in order to reduce the time of aplasia and the risk of infections during the chemotherapeutic or radiotherapeutic treatment of cancer (solid tumors) patients, during the treatment of malignant hematologic diseases, following a bone marrow transplantation (autologous or allogenic) in order to reduce the aplastic phases, to reduce leukopenia during acquired immunodeficiency syndrome (but also other infectious disorders), for the treatment of diseases involving myeloid disorders (such as

congenital agranulocytosis, aplastic anemia or myelodisplastic syndrome). The beginning of the clinical trials has evidenced the positive aspects of its use: GM CSF was found to be well tolerated and able to induce dose dependent increases in blood leukocytes counts (neutrophils but also eosinophils and monocytes). For exemple, a shortening from 7.4 to 3.5 days of the mean neutropenia duration was observed for patients submitted to a chemotherapy for solid tumors and receiving GM CSF doses from 4 to 32 microgramme/kg/day. The observed hematopoietic recoveries are unstable (less than two weeks after the end of the treatment) and for most of the patients GM CSF infusion must be extended in order to maintain a high level of leukocytes. For patients with severe idiopathic aplastic anemia important individual variations were observed: a granulocyte peak appeared in the blood between1 and 21 days (mean time 4 days) after the beginning of the treatment. A better general physical condition for the patients and a reduced frequency of infections have been often reported suggesting that GM CSF has also corrected some defects in neutrophil and monocyte functions.

Because of these effects GM CSF could be of high interest for the treatment of the hematopoietic syndrome during radiation accidental exposure.

The GM CSF has already been used in the treatment of some patients with bone marrow failure due to an accidental radiation exposure. It was concluded after the Goiania accident that by initiating GM CSF use early after exposure to near lethal doses of

radiations, the period of granulocytopenia may be shortened and survival improved.

The optimal schedule of GM CSF seems to be 5 to 15 microgramme/kg/day subcutaneously. Side effects included bone pains, chills, headhaches, mild myalgias and fever, but also for higher doses (60 microgramme/kg/day), capillary leak syndrome with generalized edema, pleural and pericardial effusion was reported as well as large vessel thrombosis. It was reported that a high dose (64 microgramme/kg/day) GM CSF assay for myelodisplastic syndrome patients had initiated rather than prevented the onset of acute myeloid leukemia.

Whether GM CSF has carcinogenic potential or will stimulation with GM CSF results in bone marrow exhaustion or stem cell failure were some of the questions that had to be solved before hemopoietic growth factors could be used on a large scale and for radiopathology purposes. Taking advantage of phase1 and 2 clinical trials as well as compasionnate need cases GM CSF cures, we have developed during this contract an experimental protocol in order to study the long term and side effects of GM CSF.

Long term bone marrow cultures (LTBMC) have provided a powerful tool to demonstrate important cell-cell interactions together with the influence of hemopoietic growth factors necessary for the maintenance of hemopoiesis.

In order to study the long term interaction between GM CSF hemopoietic cells, tumoral cells and stromal bone marrow cells, we have set up a LTBMC assay during (and after) the course of GM CSF clinical trial for treatment of leukopenia observed after autologous bone marrow transplantation in advanced solid tumor therapies. LTBMC were set up before any treatment and sequentially after each step of the protocol (double autologous bone marrow transplantation with radiotherapy, chemotherapy and GM CSF infusion) up to one year after treatment. Hemopoiesis was assessed by granulocyte macrophage progenitors (GM CFU) and mature cell production in the supernatant of the LTBMC. Stromal cells were studied for their growth and differentiation pattern. The presence of abnormal cells was studied by culture, cytological or immunological methods. Controls were assessed using bone marrow samples from the same patient before treatment and from normal donnors with matching ages.

Following a bone marrow transplantation or myelosuppressive therapy, patients with severe bone marrow failure have received GM CSF as compassionates. As the bone marrow of this kind of patients may be closer to the marrow of a radiopathology patient than the bone marrow of solid tumor patients, we have developed a LTBMC assay to study the aplastic bone marrow. Short term and side effects of the growth factor therapy were studied during this assay. The effects of repeated growth factor therapies were also investigated.

Although short term effects of GM CSF were well documented through in vitro and animal studies, when this study was initiated, the knowledges about long term in vivo effects of GM CSF on human were scarce. Therefore, we have designed a protocol to study paraclinical long term effects of GM CSF during the course of an autologous bone marrow transplantation programme, and paraclinical and clinical side effects of GM CSF during a protocol of treatment of bone marrow failure. These two main points are developed below.

2 AUTOLOGOUS BONE MARROW TRANSPLANTATION STUDY

Granulocyte-macrophage colony stimulating factor (GM CSF) is under clinical trials and shows promising effects over hematopoiesis. Disseminated neuroblastoma are treated with massive therapy followed by two autologous bone marrow transplantations (ABMT). As a small amount of malignant cells is still present in the marrow of these children after the induction treatment, neuroblastoma cells are magnetically depleted in vitro with specific monoclonal antibodies before grafting. In order to shorten granulopenia, GM CSF is administered after one of the two ABMT so that all patients received GM CSF once.
We have previously developed a long term bone marrow culture (LTBMC) system with potential application to the follow up of stage IV neuroblastoma children treated with high dose radiotherapy combined to chemotherapy followed by autologous bone marrow transplantation. The effects of in vivo GM CSF infusion over the LTBMC system were assessed during the clinical trial in order to study the interaction with neuroblastoma cells, hematopoietic and stromal bone marrow cells.

Children were diagnosed with metastatic neuroblastoma and enrolled in this prospective study. All patients were treated according to the SFOP NB 87 induction protocol and the current LMCE consolidation regimen which have been described already.

Briefly, induction chemotherapy regimen consisted in four alternating chemotherapy cycles of CADO (Cyclophosphamide 300mg/m2/day on days1to 5, Doxorubicin 60mg/m2/day on day 5, Vincristine1,5mg/m2/day on days1and 5) and VP/CIS (Etoposide 100mg/m2/day on days1to 5 and Cisplatinum 40mg/m2/day on days1 to 5) starting with a CADO cycle. Surgery was performed at the end of the induction chemotherapy prior to bone marrow harvest for graft. Autologous bone marrow was purged using an immunomagnetic depletion technique slightly modified by Lopez et al. from the method initialy described by J. Kemshead.

Consolidation regimen was performed after a predetermined plan:

-Patients in complete remission (CR) or in very good partial remission (VGPR) received the association of Vincristine, Melphalan and a 12 Grays fractionated total body irradiation (TBI) followed by autologous purged bone marrow rescue.

-Patients in partial remission (PR) underwent the double autologous bone marrow transplantation (ABMT) programme of the LMCE group consisting of a first consolidation made of BCNU 300 mg/m^2 on day 1, VM26 250 mg/m^2 on days 2 to 5, Carboplatinum 250 mg/m^2 on days 2 to 6. A second bone marrow harvest with immunomagnetic depletion was performed prior to a second consolidation made of Vincristine, Melphalan and TBI. These patients received as part of a double blind study of recombinant GM CSF 14 microgramme/kg/day as a continuous infusion of CHO material(Sandoz) during 14 days following either the first or the second ABMT (so that all patients received GM CSF and placebo once).

The timing for bone marrow sampling for the study was as follow: a sample was harvested from four different sites of the iliac bone under anesthesia at diagnosis, after induction chemotherapy (around first bone marrow harvest for ABMT), between the two grafts if applicable. The post-graft sampling was made of 7 samples harvested (when possible due to the poor condition of some of these patients) at 2, 6, 9, 12,18, 24, 28 months post TBI.

Information about long term survivors were obtained by studying the marrow from patients treated by other induction chemotherapy protocols who underwent the same single ABMT consolidation regimen as CR patients.

Patients were between 2 and 4 years old. Ten hematologically normal patients (stage 1 neuroblastoma patients with comparable ages), normal bone marrow samples from healthy allogenic bone marrow transplantation donors were also investigated as controls.

Long term bone marrow culture study (LTBMC) were as follow. The sample (about 2 ml over heparine-Roche) was diluted (1/3) with Iscove's medium (Gibco) and centrifuged over ficoll of density 1.119 (Sigma) 10 minutes at 200 g followed by 15 minutes at 400 g to get rid of most of the red cells. The nucleated overlaying cells were washed three times (10 minutes at 400 g) in Iscove's medium 2% foetal calf serum (FCS-Boehringer) and

cultured (5% CO_2, at 33°C) after the viability of the cells had been assessed by the trypan blue exclusion test (2.10^6 viable cells/ml in Iscove's medium 10 % FCS, 10% horse serum - Boehringer, 10^{-6} M hydrocortisone hemisuccinate - Sigma). Half of the supernatant was removed each week, the collected cells were counted (expressed in million cells per millilitre, C/ml) and the clonogenicity of granulocyte-macrophage progenitors (GM CFU) was tested in triplicate among the non adhering cells using conditioned medium of the human 5637 cell line as a source of hemopoietic colony stimulating factors (10^5 cells/ml in Iscove's medium, 0.3 % agar -Difco, 20% conditioned medium of 5637 cell line, 15 % FCS, cultured in Petri dishes at 5% CO_2, 37°C). The presence of preadipocytic cells identified among the stromal cells by fat inclusions (Fluorescence of Nile red dye -Aldrich, 10 mg/ml, excitation wavelength: 450-500 nm, emission wavelength >580 nm) was also monitored.

Cultures in agar without conditioned medium from 5637 cells were also set up in order to test the growth of other cell types (i.e. tumor cells). All the semi-solid cultures were scored on day 11, the colonies being defined as groups of more than 50 cells, clusters as groups of 10 to 50 cells. The mean counts of three Petri dishes containing GM CFU colonies was expressed as the quantity of clonogenic cells to the number of viable cells harvested per millilitre of LTBMC supernatant, on the day of collection and agar culture (GM CFU/ml).

Supernatant samples from LTBMC were cytocentrifuged (10 minutes at 200 g) on glass slides. The cells were stained to evaluate the presence of tumor cells in the culture. Three dimensional out growth of potential tumor cells occured in the cultures established from patients with advanced bone marrow metastatic neuroblastoma. They were removed from the stroma layer in some of the flasks of each cultured sample by flushing with medium and subcultured for 4 to 8 weeks, in order to initiate spheroid growth (Iscove's medium, 15% FCS, 0.2% agar at 5 % CO_2, 37°C). The spheroids of cells obtained were freezed down or included in parafin and processed for histological analysis and/or assayed for neurone specific enolase (NSE) or anti GD2 response using enzymo-immunological assays (DAKO). Due to the large variation of results observed between various batches of serum, all the horse and fetal calf serum used have been preselected through long term and short term cultures of normal human or mouse bone marrow and the same batches has been used during all the length of the study.

Among the 12 studied patients only 5 have completed the protocol. The semilogarithmic scaled figures show the quantity of cells and the pattern of clonogenicity of the GM CFU among supernatant cells during the time course of the cultures. Results of clonogenicity experiments using colonies counts or cluster plus colonies counts are comparable. In most of the experiments of secondary semi-solid culture from neuroblastoma patients bone marrow cells, as

well as some colonies or clusters of tumor cells may be evidenced due to their peculiar morphology.

The quantity of viable cells and the clonogenicity of the GM CFU among supernatant cells during the LTBMC were followed for at least one month. The settling tumor cells could be subcultured and characterized as spheroids. The interactions between neuroblastoma cells and the bone marrow stroma could be evidenced through the culture, histological and immunocytological data. When available fresh stromal layer where also investigated. The clinical trials of GM CSF have already evidenced its usefulness in the treatment of granulopenia, however long lasting effects on other cells (stromal cells or tumoral cells) are not well documented. The human LTBMC assay we have developed demonstrate the production of hemopoietic stem cells for several weeks and might be of diagnostic and prognostic value to assess neuroblastoma invasivness and the functions of the bone marrow during treatment. During this assay no long term effect of GM CSF was evidenced when comparing ABMT and placebo versus ABMT and GM CSF for each patient

(figures 1a to 5a: Cellularity in LTBMC from patient 1-5, figures 1b to 5b: Clonogenicity in LTBMC from patient 1-5). The long term bone marrow cultures of the neuroblastoma patients are impaired in terme of cellularity and clonogenicity when compared to stage I neuroblastoma patients with comparable age as controls (figures 6, 7, 8, 9: Mean cellularity in LTBMC from patients at diagnostic, Mean clonogenicity in LTBMC from patients at diagnostic, Mean cellularity in LTBMC from controls, Mean clonogenicity in LTBMC

from controls). The results of the cultures for patients who did not received GM CSF and for the patients who received it are very similar suggesting that the treated patients do not evidence long term effect of GM CSF on the bone marrow (figures 10, 11, 12, 13: Mean cellularity in LTBMC from patients after ABMT, Mean cellularity in LTBMC from patients after ABMT and GM CSF,Mean clonogenicity in LTBMC from patients after ABMT, Mean clonogenicity in LTBMC from patients after ABMT and GM CSF). LTBMC from normal bone marrow donors give very similar results to LTBMC from stage I neuroblastoma controls. Late survey (more than 10 months after total body irradiation and up to 28 months) do not evidence a clear GM CSF effect either, although the LTBMC evidence the long lasting impairment of the marrow functions following the therapies (figures 14, 15, 16, 17: Late survey of clonogenicity in LTBMC after ABMT without GM CSF, Late survey of cellularity in LTBMC after ABMT and GM CSF, Late survey of cellularity in LTBMC after ABMT without GM CSF, Late survey of clonogenicity in LTBMC after ABMT and GM CSF).

No secondary diseases have appeared for these patients. No bone marrow exhaustion related to GM CSF treatment was reported. We did not evidenced any effect of GM CSF on preadipocytes among stromal cells during long term follow up.

Cloning and growth of neuroblastoma cells in semi-solid media have been reported previously and the fundamental importance of the interaction between tumor cells and hemopoietic function for diagnosis, prognosis and follow up has been emphasized. We did not evidence any action of GM CSF either on the number of clonogenic

tumoral cells,which may be representative of the quantitative growth potential of the tumor, or on the spheroid subcultures, which may be closer to the typical growth behavior of the tumors. Histochemical stainings, cytological examinations and immunoenzymatic assays for the presence of NSE and GD2 did not evidence any change in the differentiation pattern of neuroblastoma cells following growth factor therapy. The observed normal initial hemopoietic cell production already documented suggests that the interaction between impaired bone marrow function and neuroblastoma cell growth is not a direct inhibition of the hemopoietic stem cell production.

Results evidence no long term effect of GM CSF infusion when compared to control either on normal (hematopoietic or stromal) cells or on tumoral cells. The assay we developed might be of diagnostic and prognostic value to assess the functions of the bone marrow during treatment and to assess the effect of hematopoietic growth factors. During the GM CSF clinical trial for solid tumor patients, at the level of LTBMC, we have not evidenced adverse or positive effects of GM CSF on stromal cells. The GM CFU clonogenicity assays and the production of mature cells in LTBMC supernatant are not modified either. Abnormal cells do not appear more frequently suggesting that in this peculiar disease the tumoral cells are not sensitive to the growth factor. No abnormal bone marrow cells was evidenced by culture technique. This results suggest that, in this protocol, GM CSF has no long term adverse effects on the bone marrow.

3 HEMATOPOIETIC GROWTH FACTOR TREATMENT OF SECONDARY APLASIA STUDY

GM CSF was used as a treatment in 21 compassionate need cases. A study of short term side effects and in vitro effects of the growth factor was set up.

Patients had marrow failure (less than 500 blood leukocytes/mm^3) following allogenic bone marrow transplantation for the treatment of aplastic anemia, chronic or acute leukemias (the conditionning regimen included a 10 Gy single exposure total body irradiation) and was associated with graft versus host disease (11 patients), cytomegalovirus or other viral infections (10 patients), veino occlusive disorder (in one case), aspergillosis or candidosis (3 patients). Patients received a first cure of 0.216 mg per day for 14 days subcutaneously. The treatment was continued when needed and well tolerated. Vital signs monitored were weight, systolic pressure, diastolic pressure, radial pulse, respiratory rate, body temperature and maximal temperature. Karnofsky performance was scored. Hematology included blood leukocytes, erythrocytes, platelets, reticulocytes, eosinophils, monocytes, lymphocytes, basophils, neutrophils, hemoglobin, hematocrit. Biochemistry included measurement of Sodium, Potassium, Creatinine, Urea, Calcium, Total Bilirubin, ASAT (GOT), ALAT (GPT), Gamma GT, Alkaline phosphatase, Total Cholesterol, Total Protein, Albumine, Globuline, Uric Acid, Glucose. Overall tolerability and efficacy was

correlated to the concomitant medications and assessed by investigators. Attempt to established long term bone marrow cultures for some of these patients and for patients with severe aplastic anemia patients who did not received GM CSF were realised. The sample (about1 ml over heparine-Roche) was diluted (1/3) with Iscove's medium (Gibco) and centrifuged over ficoll of density 1.119 (Sigma) 10 minutes at 200 g followed without stop by 15 minutes at 400 g to get rid of most of the red cells. The nucleated overlaying cells were washed three times (10 minutes at 400 g) in Iscove's medium 2% foetal calf serum (FCS-Boehringer) and cultured (5% CO_2, at 33°C) after the viability of the cells had been assessed by the trypan blue exclusion test (2.10^6 viable cells/ml in Iscove's medium 10 % FCS, 10% human AB serum - CNTS, 10^{-6} M hydrocortisone hemisuccinate - Sigma). Half of the supernatant was removed each week, the collected cells were counted (expressed in million cells per millilitre, C/ml) and the clonogenicity of granulocyte-macrophage progenitors (GM CFU) was tested in triplicate among the non adhering cells using conditionned medium of the human 5637 cell line as a source of hemopoietic colony stimulating factors (10^5 cells/ml in Iscove's medium, 0.3 % agar - Difco, 10% conditioned medium of 5637 cell line, 15 % FCS, cultured in Petri dishes at 5% CO_2, 37°C).The main probleme encounter was the very low quantity of cells in the sample (we had to use Leighton tubes or 10 square centimeters dishes in order to keep a high concentration of cells in the culture as attempt to culture the cells at lower density were

unsuccessful) and its sensitivity to the toxic effects of horse serum.

Overall tolerability was good or very good in 17 cases, moderate in 4 cases, no poor tolerability was reported. Efficacy was good or very good in 12 cases, moderate in 5, slight in one case and no efficacy at all was reported in 3 cases. The large heterogeneity of the indications for compassionate need case do not allow to draw a general conclusion from this study. However it appears that the dose used during this assay (which was about 8 microgrammes/kg/day) was well tolerated and had a positive role on leukopenia in most patients as evidenced by the results of the hematology counts
(figures 18, 19, 20, 21, 22: Mean leukocytes counts during GM CSF treatment, Mean erythrocytes counts during GM CSF treatment, Mean platelets counts during GM CSF treatment, Mean hemoglobin level during GM CSF treatment, Mean hematocrit during GM CSF treatment) The observed changes on platelets and red cells counts correlate with transfusion of these elements to the patients and are not likeky to be due to a direct positive role of GM CSF on these cell lines. When a patient received more than one treatment it appear that there was no long lasting effects on leukocytes blood counts and that the effects of the cure were not really cumulative although some patients had higher leukocyte level during the repeated cures (figures 23a to 23e: Leukocytes counts in GM CSF treatments of five patients). Cell production exhaustion was not reported in response to GM CSF therapy although the given

dose had to be enhanced for some patients in an attempt to reach a better efficacy (up to 0.638 mg per day). The growth factor therapy does not seems to act on the other studied parameters. Study on bone marrow failure using a long term bone marrow culture technique has laid to unclear results: we have been able to developpe an assay for aplastic bone marrow (using human AB serum instead of the classical horse serum used in LTBMC, a stromal layer was established in a majority of the cultured samples from severe aplastic anemia patients) but the important individual variability and the poor condition of the patients has not allowed us to evidence any gain or loss from GM CSF with the techniques we used. We concluded that this type of culture is inappropriate for those patients.

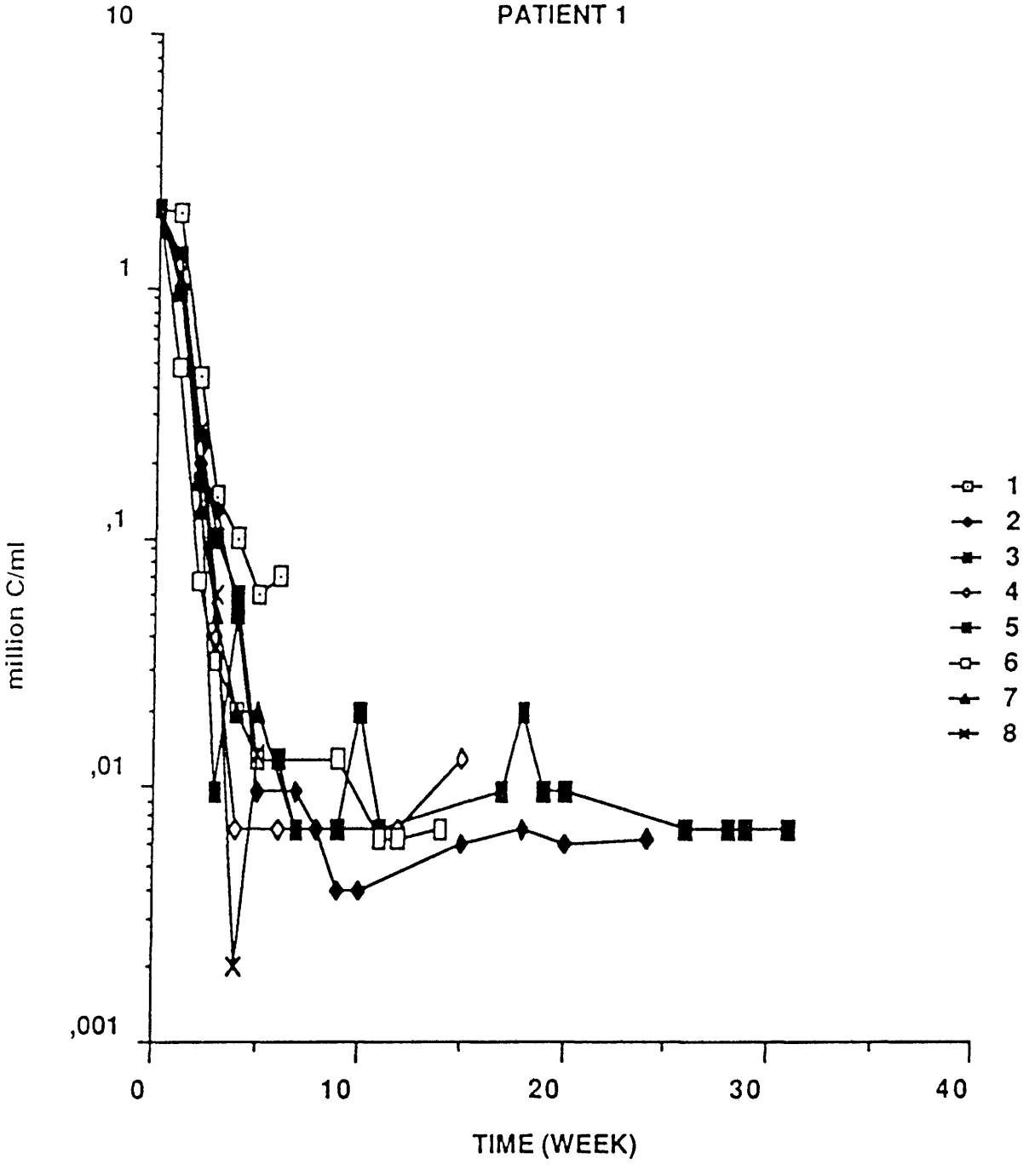
4 CONCLUSION

Although a large variety of clinical trials have been set up, the efficiency of human recombinant GM CSF therapy in the treatment of leukopenia is not well established yet. Our results suggest that there is no contraindication to the use of GM CSF for the treatment of leukopenia due to adverse long term effect of GM CSF or because of the rather mild side effects reported during these studies.

Most of the initial studies were performed in vitro and in vivo with glycosylated GM CSF produced by gene expression in eukaryotic cells (namely chinese hamster ovary cells). However the GM CSF now available for clinical trials is obtained from prokaryotic strain (E. coli) and thus non glycosylated. In vitro studies have shown that non glycosylated growth factors (except erythropoietin which is highly glycosylated in its natural form) have comparable activities to glycosylated forms. However, the presence of antibodies to non glycosylated GM CSF has been observed in the serum of treated patients. The selective immunization could hinder the efficacy of the treatment. Furthermore, a lower efficacy for blood hematopoietic stem cell harvesting (for autologous blood stem cell transplantation) has been recently reported. The differences observed between in vivo effects of non glycosylated versus glycosylated growth factors suggest that further studies are needed.

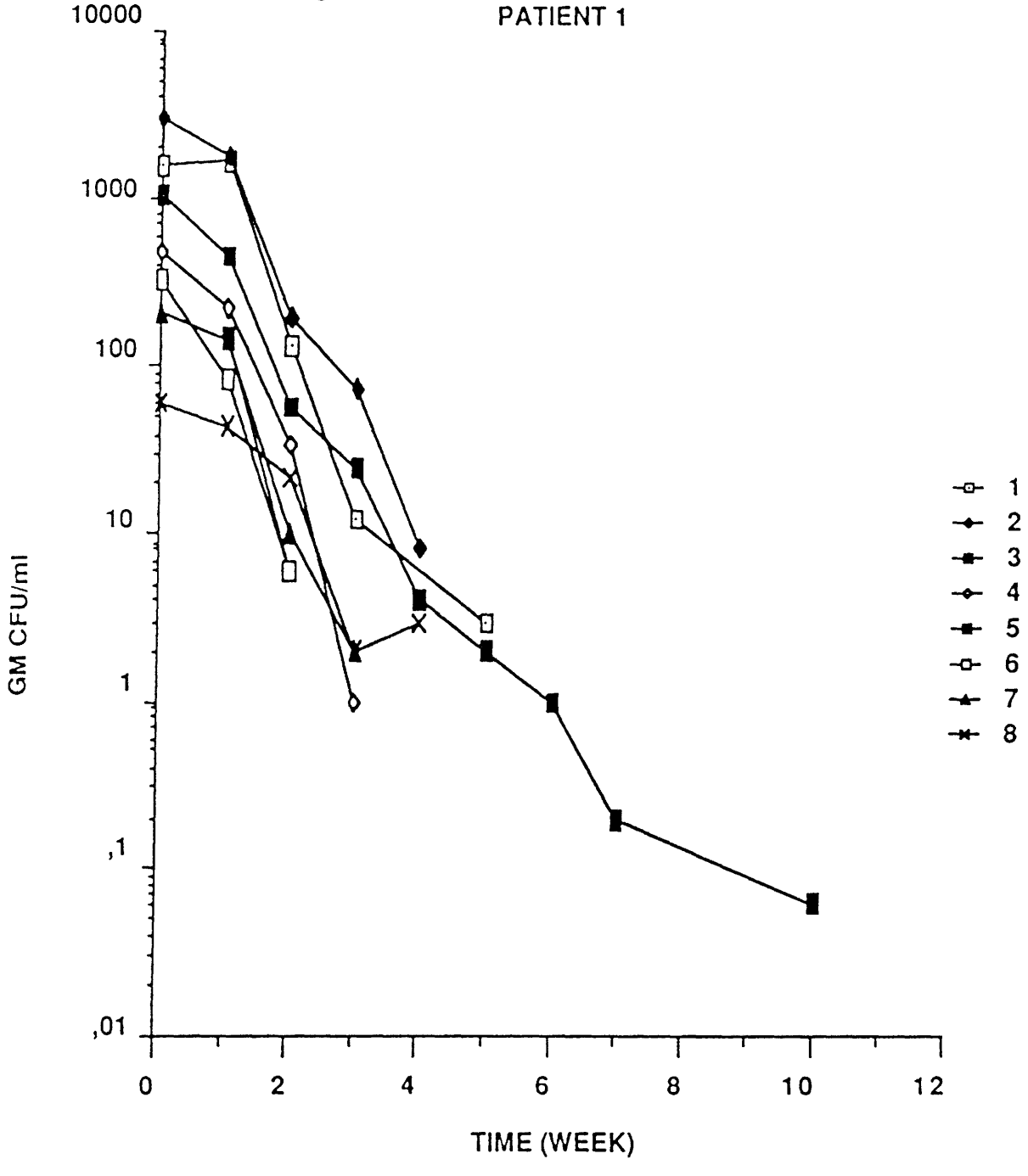
Granulocytes colony stimulating factor (G CSF) and interleukin 3 are promising hemopoietic growth factors entering clinical trial for the treatment of aplasia. Other growth factors are going to be on clinical trial and some of their properties may be important for radiopathology patients (such as interleukin 1 which may have a radioprotective effect, interleukin 6 which may act on primitive stem cells, TGF β which protect experimental animals against sepsis). Although their side effects are rather well identified, long term effects are not known, and may be of peculiar importance for IL 3 which is known to stimulate the growth of some leukemia cell lines. Furthermore clinical trials for combination of growth factors (IL 3 plus GM CSF for example) are going to be set up and the side and long term effects are not well documented. The assays we have developed will be adapted to the new protocols in order to assess their long term effects. New other assays should be developed in order to study the potential use of these factors for overexposed patients.

fig. 1a CELLULARITY IN LTBMC FROM PATIENT 1



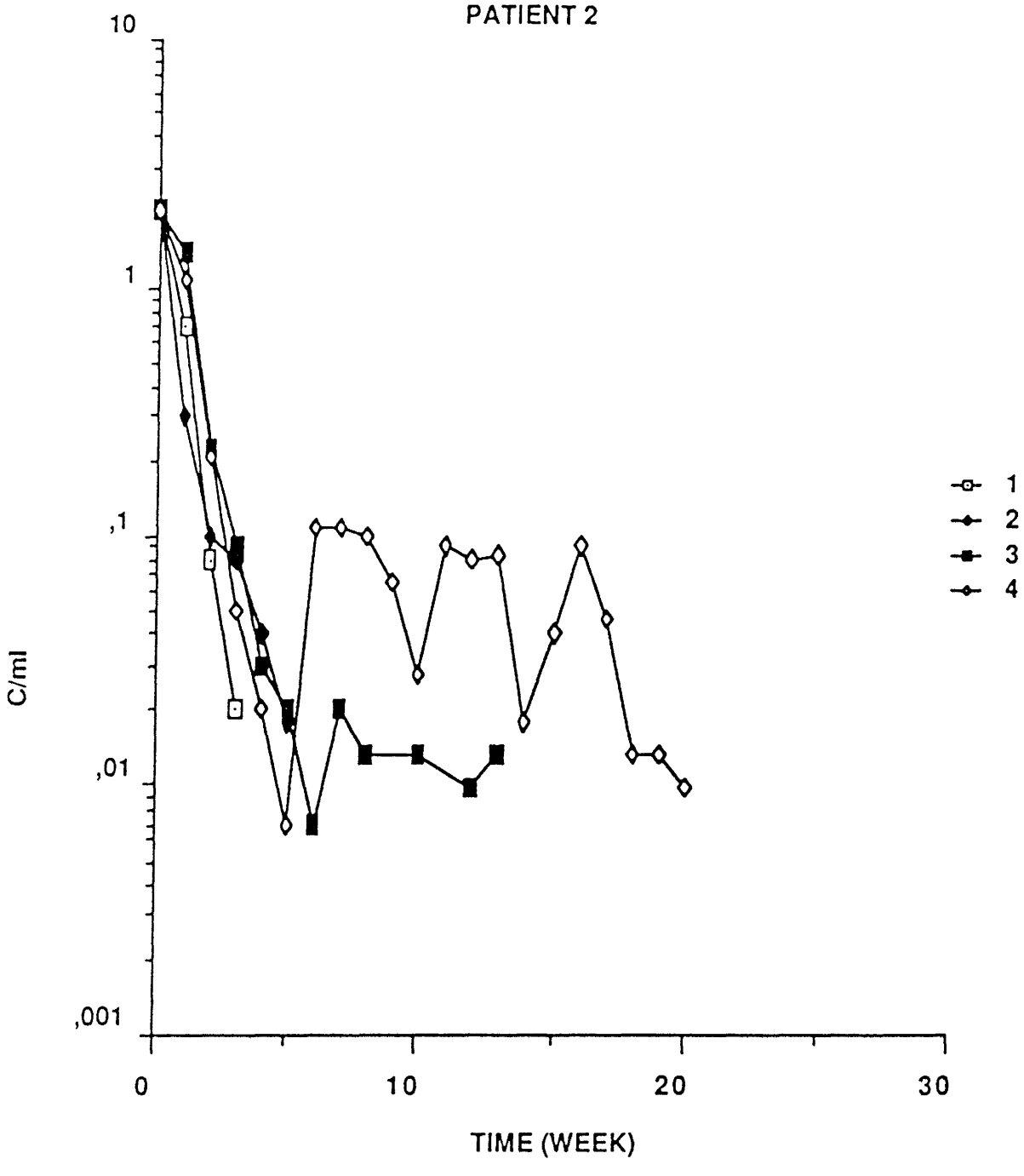
1 DIAGNOSIS
2 AFTER CHEMOTHERAPY
3 2 MONTH POST FIRST ABMT AND PLACEBO
4 1 WEEK BEFORE SECOND ABMT
5 3 WEEKS AFTER SECOND ABMT AND PLACEBO
6 3 MONTH AFTER SECOND ABMT
7 8 MONTH AFTER SECOND ABMT
8 14 MONTH AFTER SECOND ABMT

fig. 1b CLONOGENICITY IN LTBMC FROM PATIENT 1



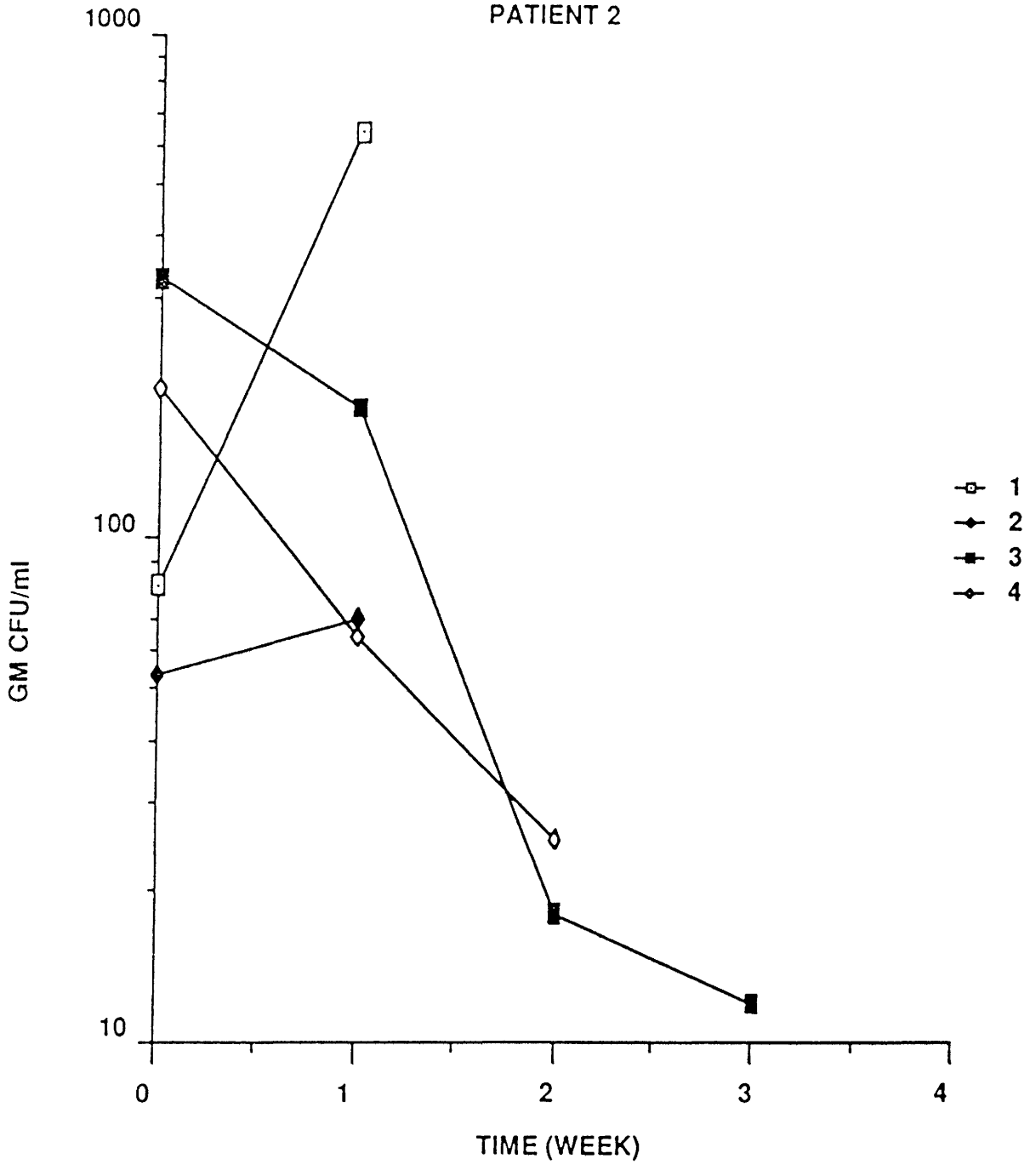
1 DIAGNOSIS
2 AFTER CHEMOTHERAPY
3 2 MONTH POST FIRST ABMT AND PLACEBO
4 1 WEEK BEFORE SECOND ABMT
5 3 WEEKS AFTER SECOND ABMT AND PLACEBO
6 3 MONTH AFTER SECOND ABMT
7 8 MONTH AFTER SECOND ABMT
8 14 MONTH AFTER SECOND ABMT

fig. 2a CELLULARITY IN LTBMC FROM PATIENT 2



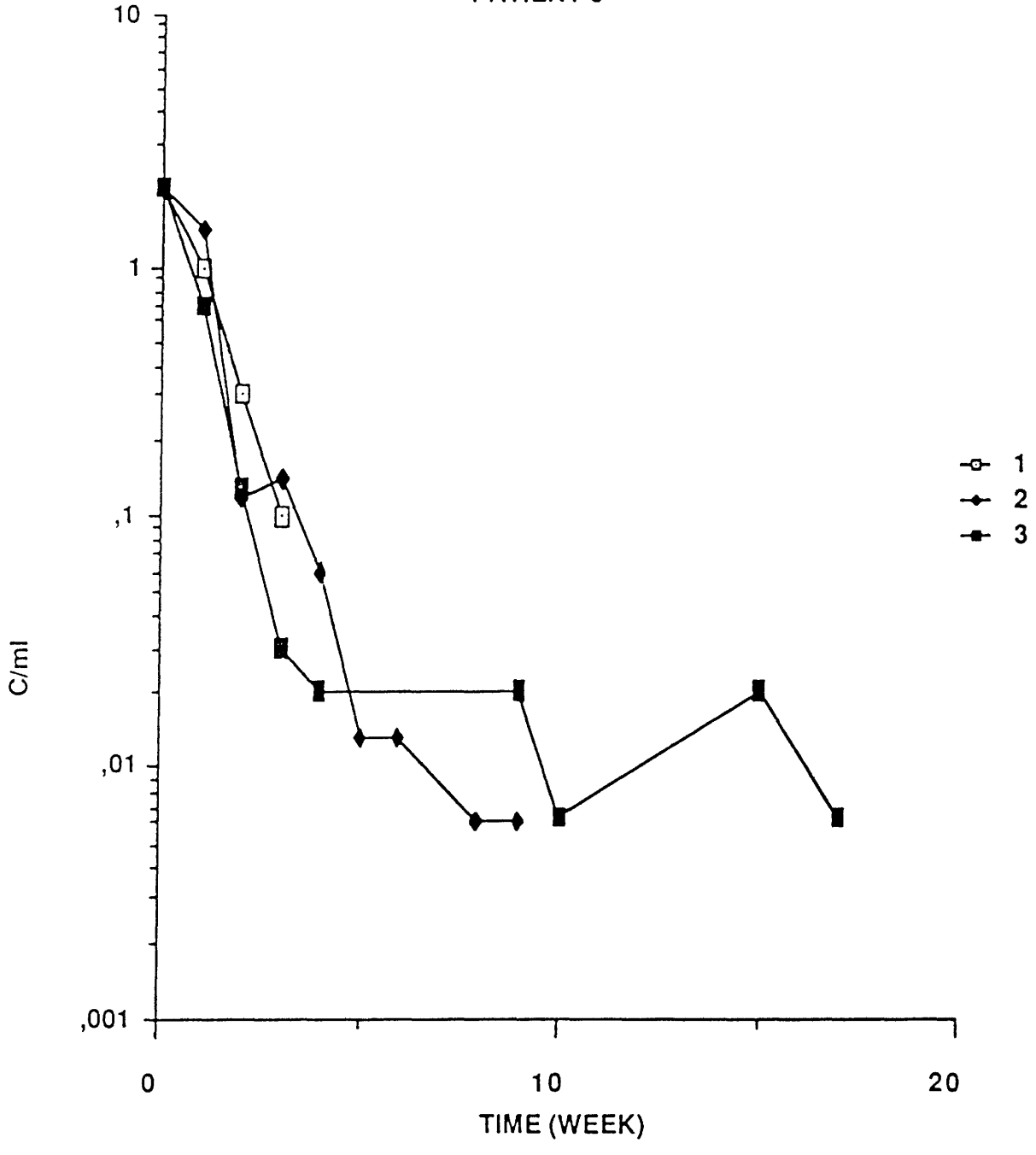
1 AT DIAGNOSIS
2 AFTER CHEMOTHERAPY
3 7 WEEKS AFTER FIRST ABMT AND GM CSF
4 8 WEEKS AFTER SECOND ABMT AND PLACEBO

fig. 2b CLONOGENICITY IN LTBMC FROM PATIENT 2



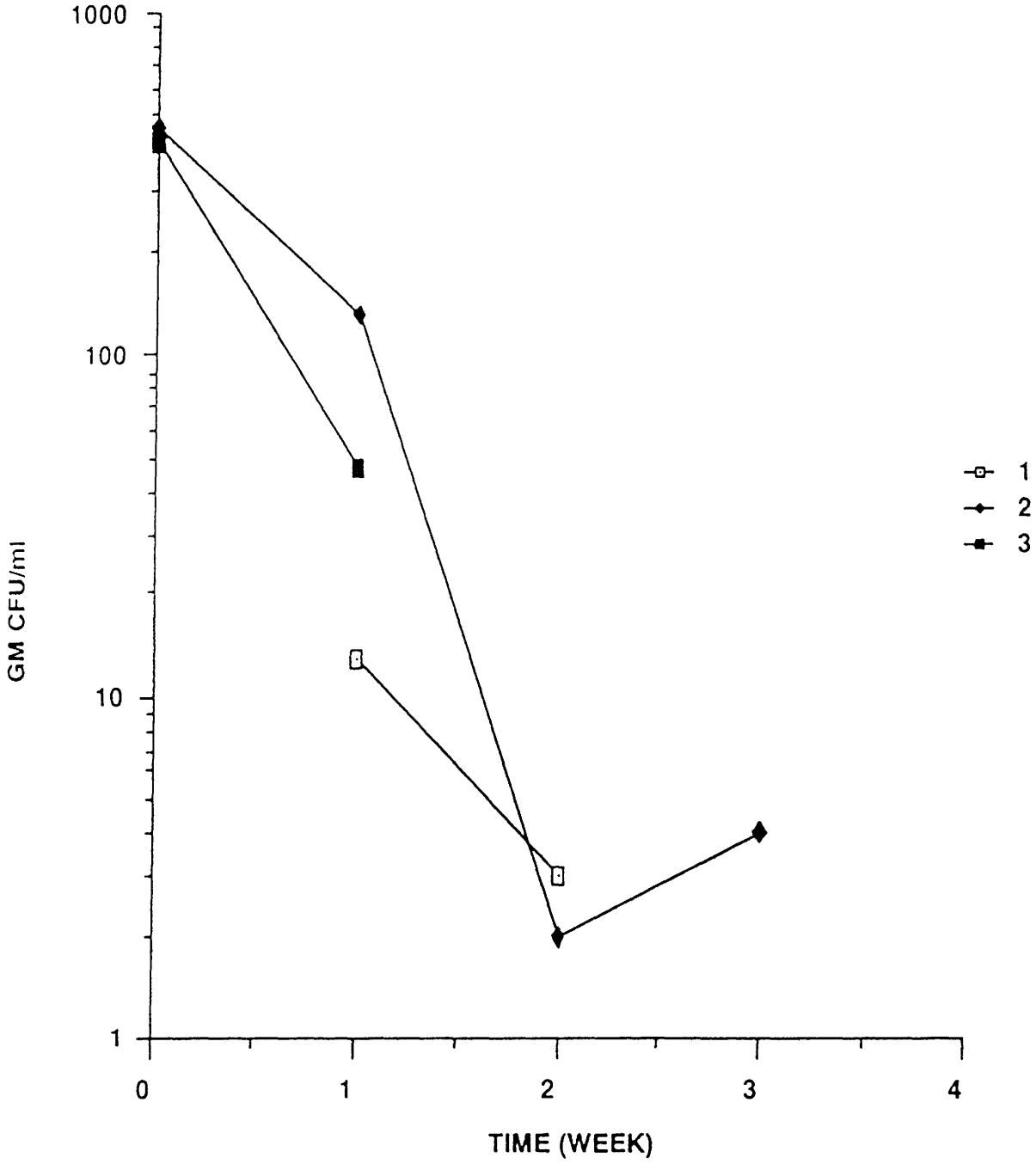
1 AT DIAGNOSIS
2 AFTER CHEMOTHERAPY
3 7 WEEKS AFTER FIRST ABMT AND GM CSF
4 8 WEEKS AFTER SECOND ABMT AND PLACEBO

fig. 3a CELLULARITY IN LTBMC FROM PATIENT 3



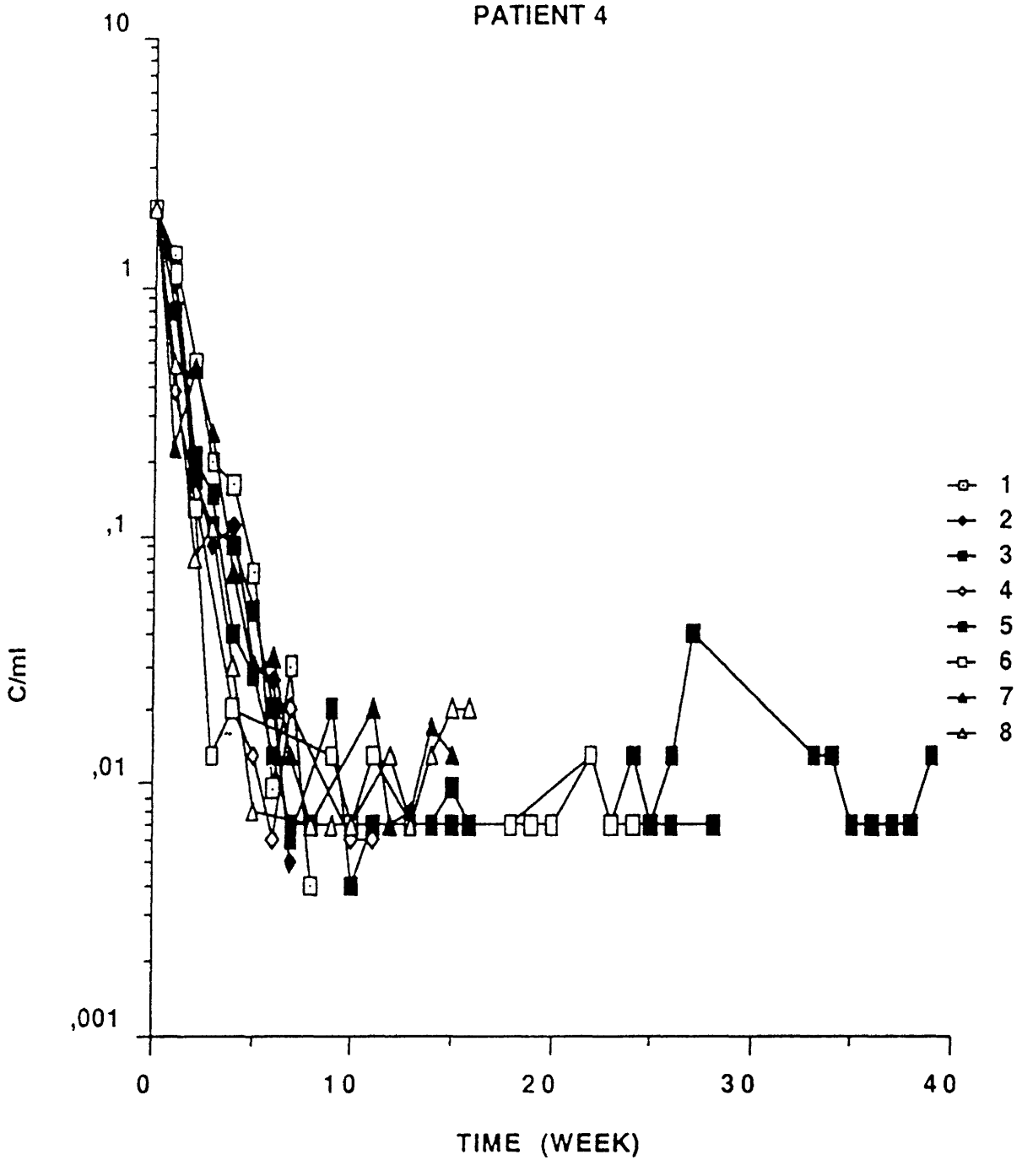
1 AFTER CHEMOTHERAPY
2 6 WEEKS AFTER FIRST ABMT AND PLACEBO
3 12 WEEKS AFTER SECOND ABMT AND GM CSF

fig. 3b CLONOGENICITY IN LTBMC FROM PATIENT 3



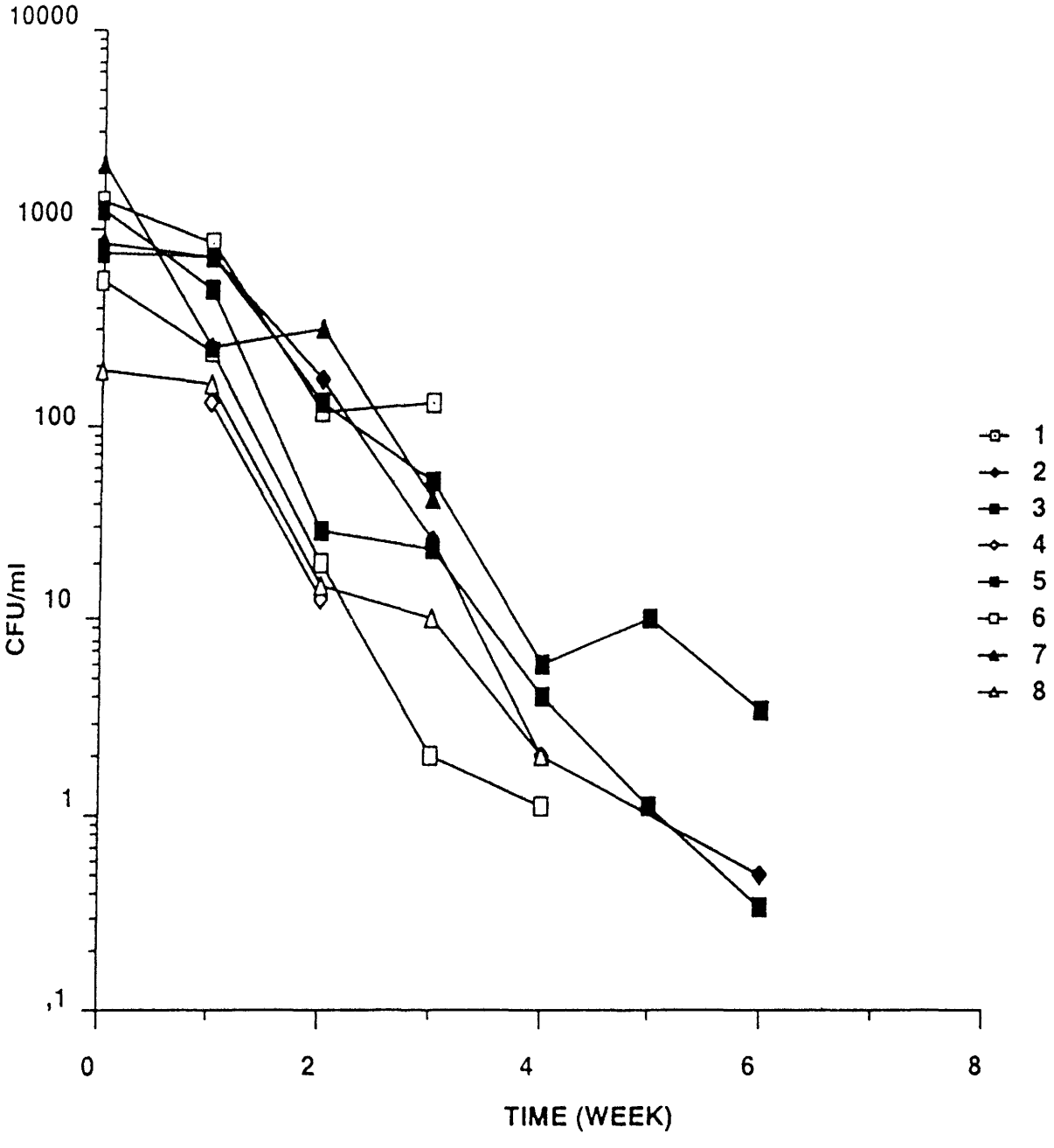
1 AFTER CHEMOTHERAPY
2 6 WEEKS AFTER FIRST ABMT AND PLACEBO
3 12 WEEKS AFTER SECOND ABMT AND GM CSF

fig. 4a CELLULARITY IN LTBMC FROM PATIENT 4



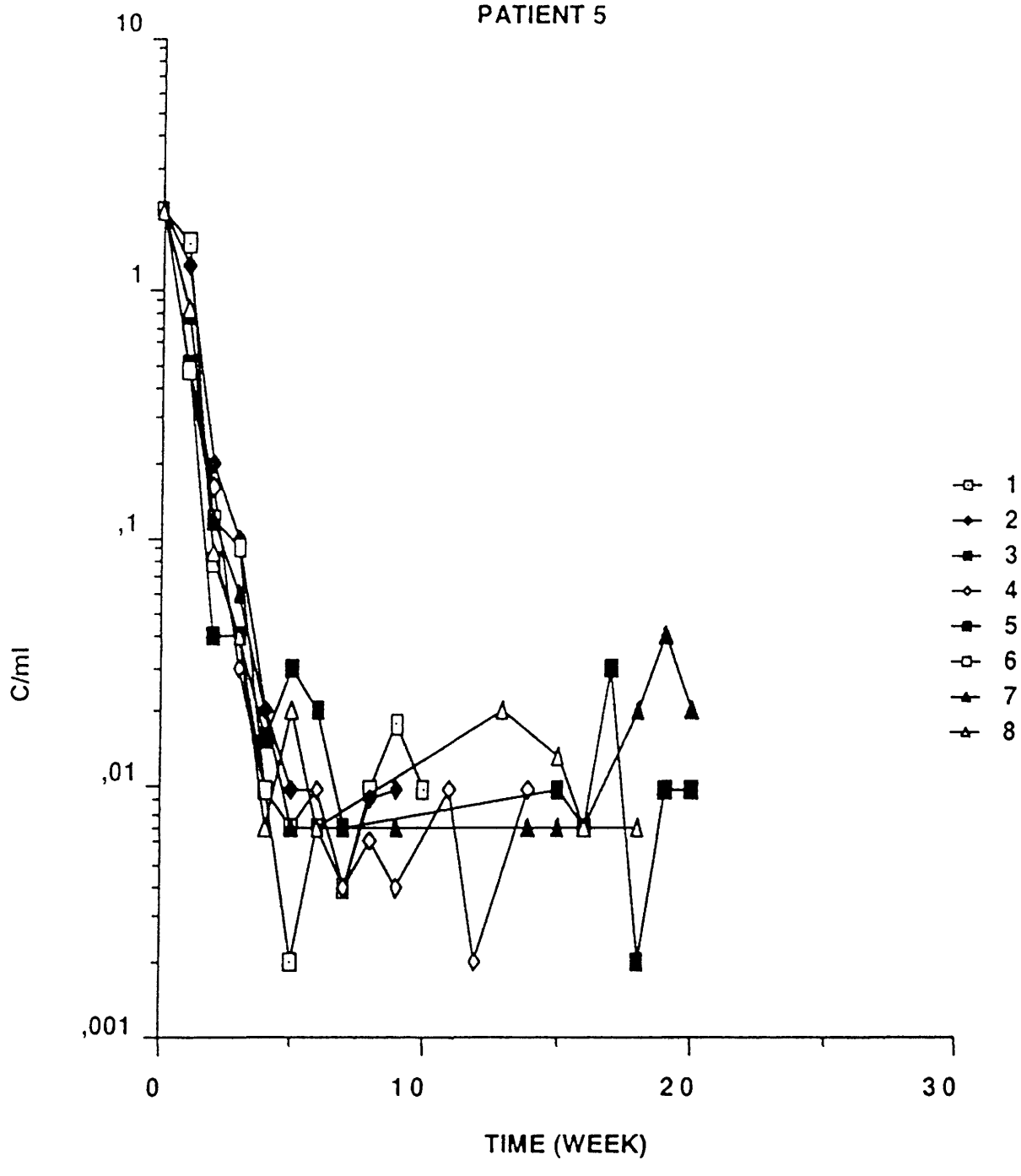
1 AT DIAGNOSTIC
2 BONE MARROW BEFORE PURGING
3 AFTER CHEMOTHERAPY
4 3 WEEKS POST FIRST ABMT AND PLACEBO
5 8 WEEKS AFTER ABMT
6 BEFORE SECOND ABMT
7 2 WEEKS POST SECOND ABMT AND GM CSF
8 8 WEEKS POST ABMT

fig. 4b CLONOGENICITY IN LTBMC FROM PATIENT 4



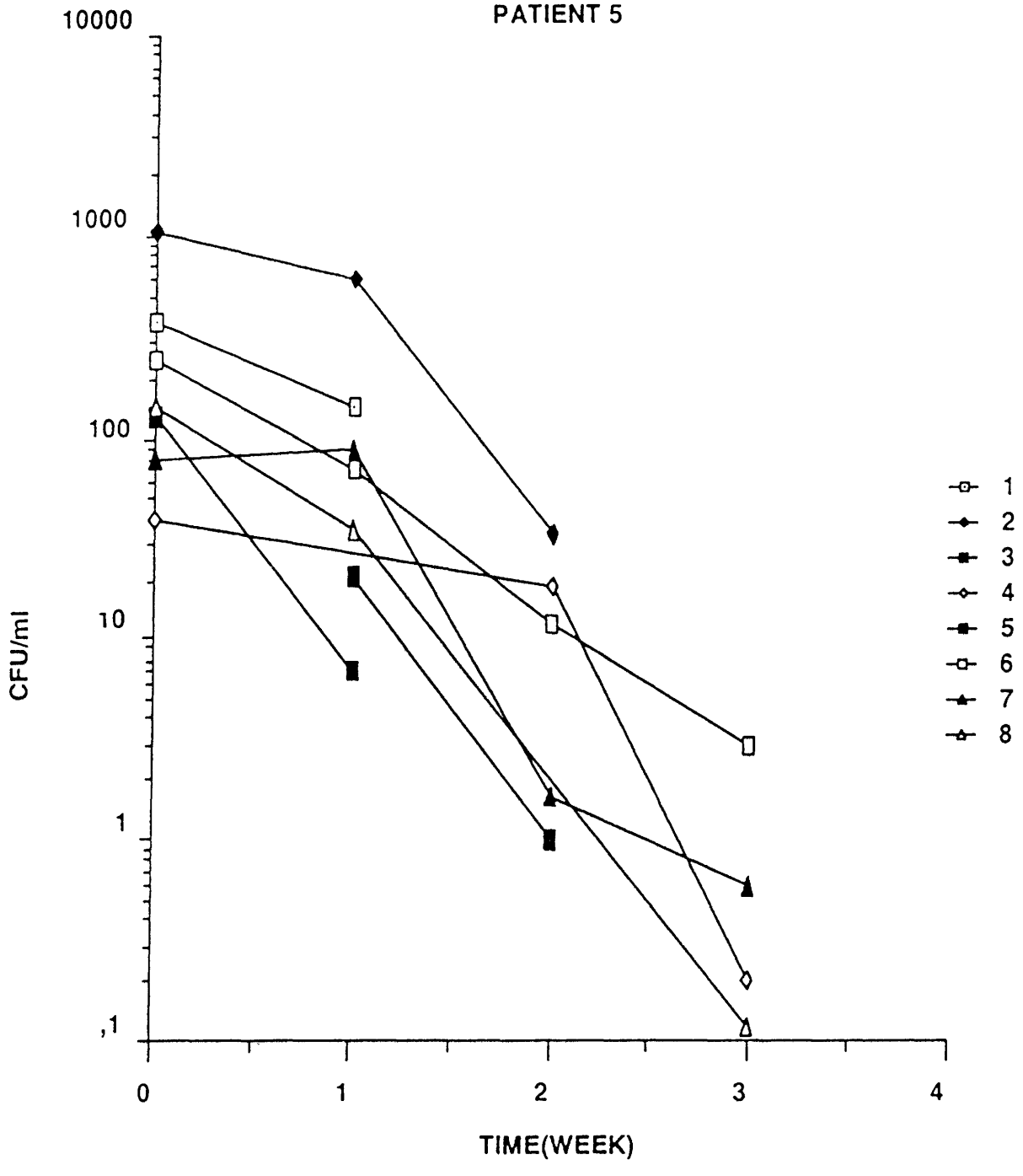
1 AT DIAGNOSTIC
2 BONE MARROW BEFORE PURGING
3 AFTER CHEMOTHERAPY
4 3 WEEKS POST FIRST ABMT AND PLACEBO
5 8 WEEKS AFTER ABMT
6 BEFORE SECOND ABMT
7 2 WEEKS POST SECOND ABMT AND GM CSF
8 8 WEEKS POST ABMT

fig. 5a CELLULARITY IN LTBMC FROM PATIENT 5



1 AT DIAGNOSTIC
2 AFTER CHEMOTHERAPY
3 BEFORE ABMT
4 10 WEEKS AFTER FIRST ABMT AND PLACEBO
5 8 WEEKS AFTER SECOND ABMT AND GM CSF
6 16 WEEKS AFTER SECOND ABMT
7 24 WEEKS AFTER SECOND ABMT
8 44 WEEKS AFTER SECOND ABMT

fig. 5b CLONOGENICITY IN LTBMC FROM PATIENT 5



1 AT DIAGNOSTIC
2 AFTER CHEMOTHERAPY
3 BEFORE ABMT
4 10 WEEKS AFTER FIRST ABMT AND PLACEBO
5 8 WEEKS AFTER SECOND ABMT AND GM CSF
6 16 WEEKS AFTER SECOND ABMT
7 24 WEEKS AFTER SECOND ABMT
8 44 WEEKS AFTER SECOND ABMT

fig. 6 MEAN CELLULARITY IN LTBMC FROM PATIENTS AT DIAGNOSTIC

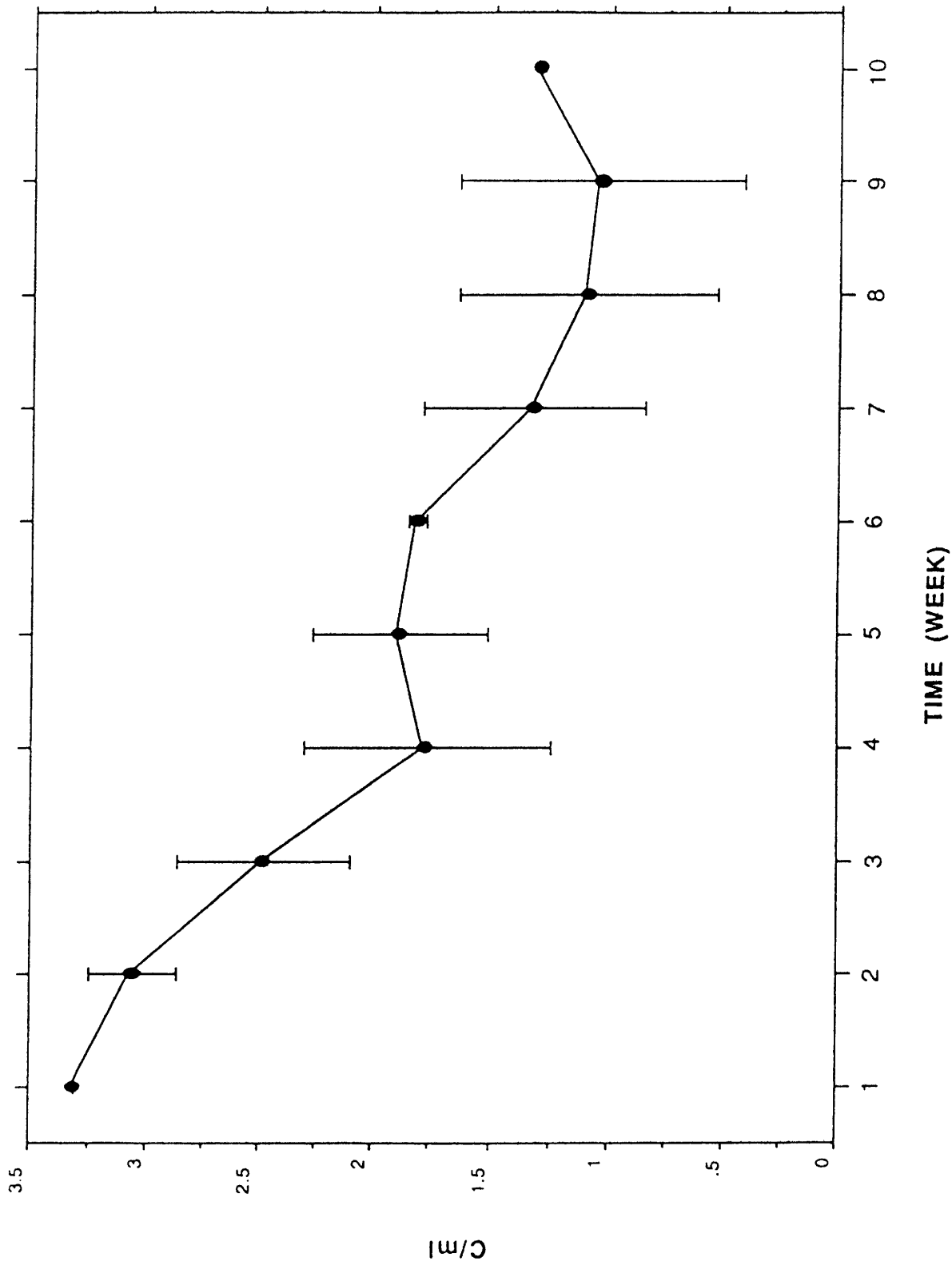


fig. 7 MEAN CLONOGENICITY FROM PATIENTS AT DIAGNOSTIC

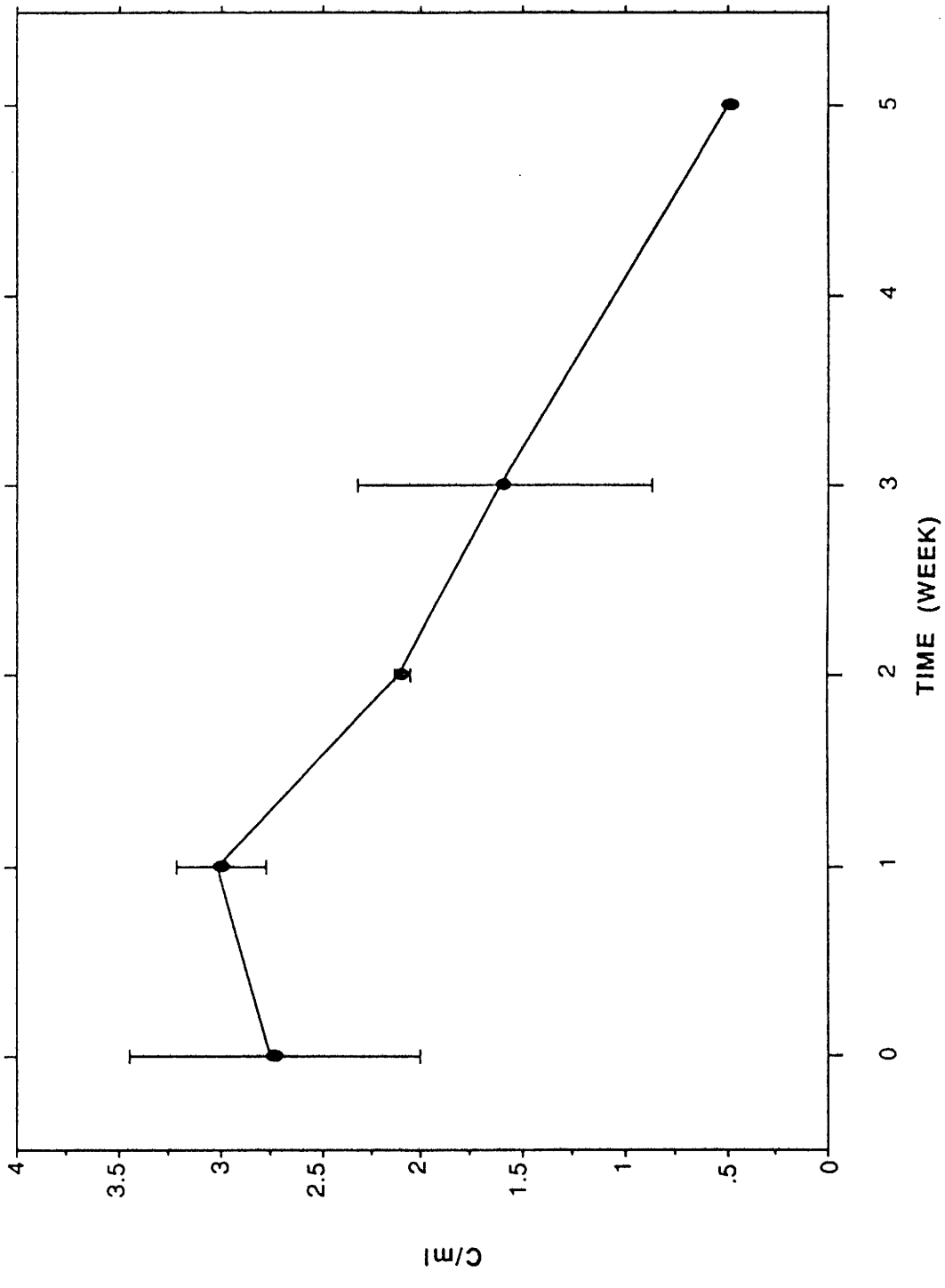


fig. 8 MEAN CELLULARITY IN LTBMC FROM CONTROLS

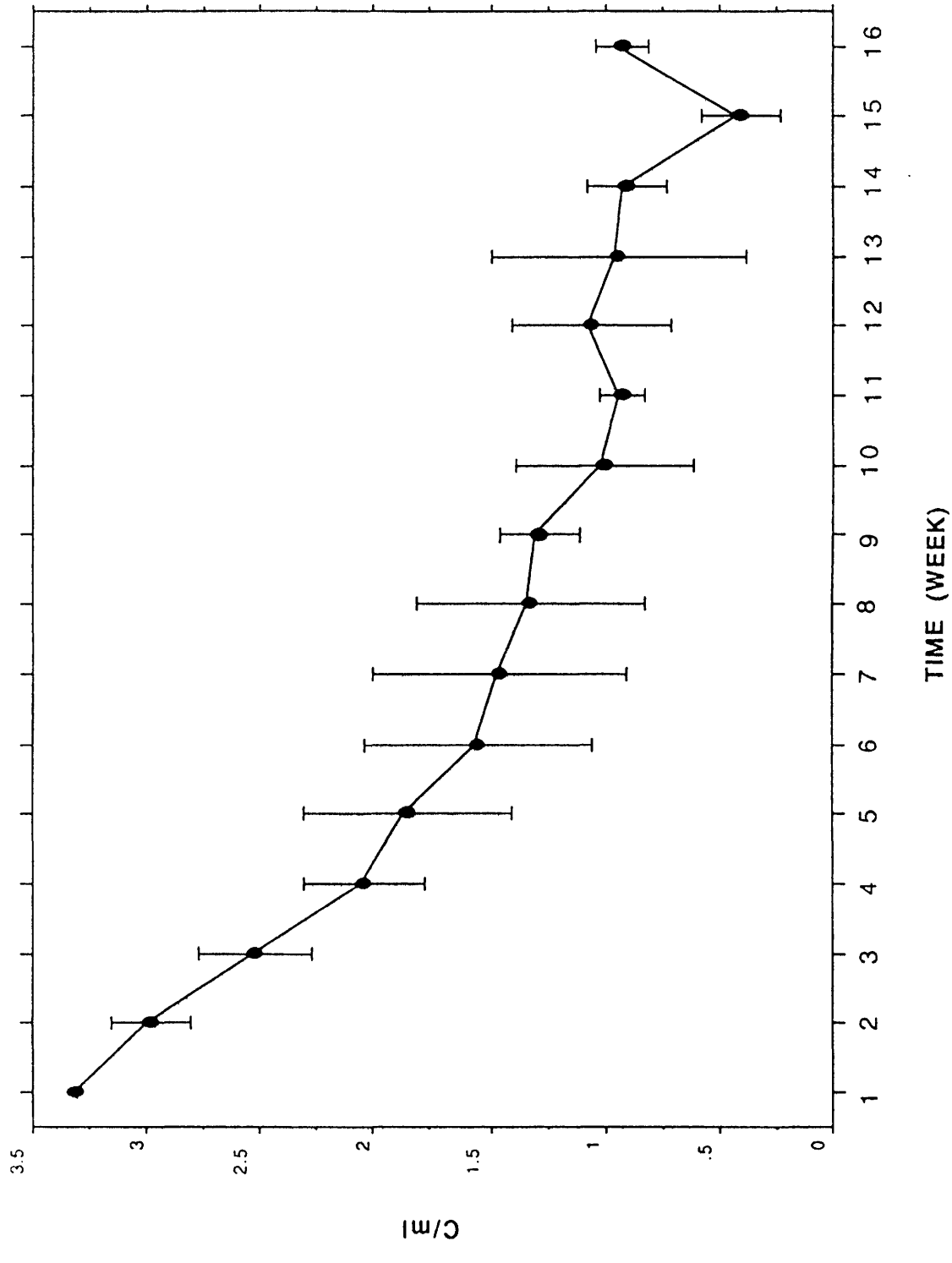


fig. 9 MEAN CLONOGENICITY IN LTBMC FROM CONTROLS

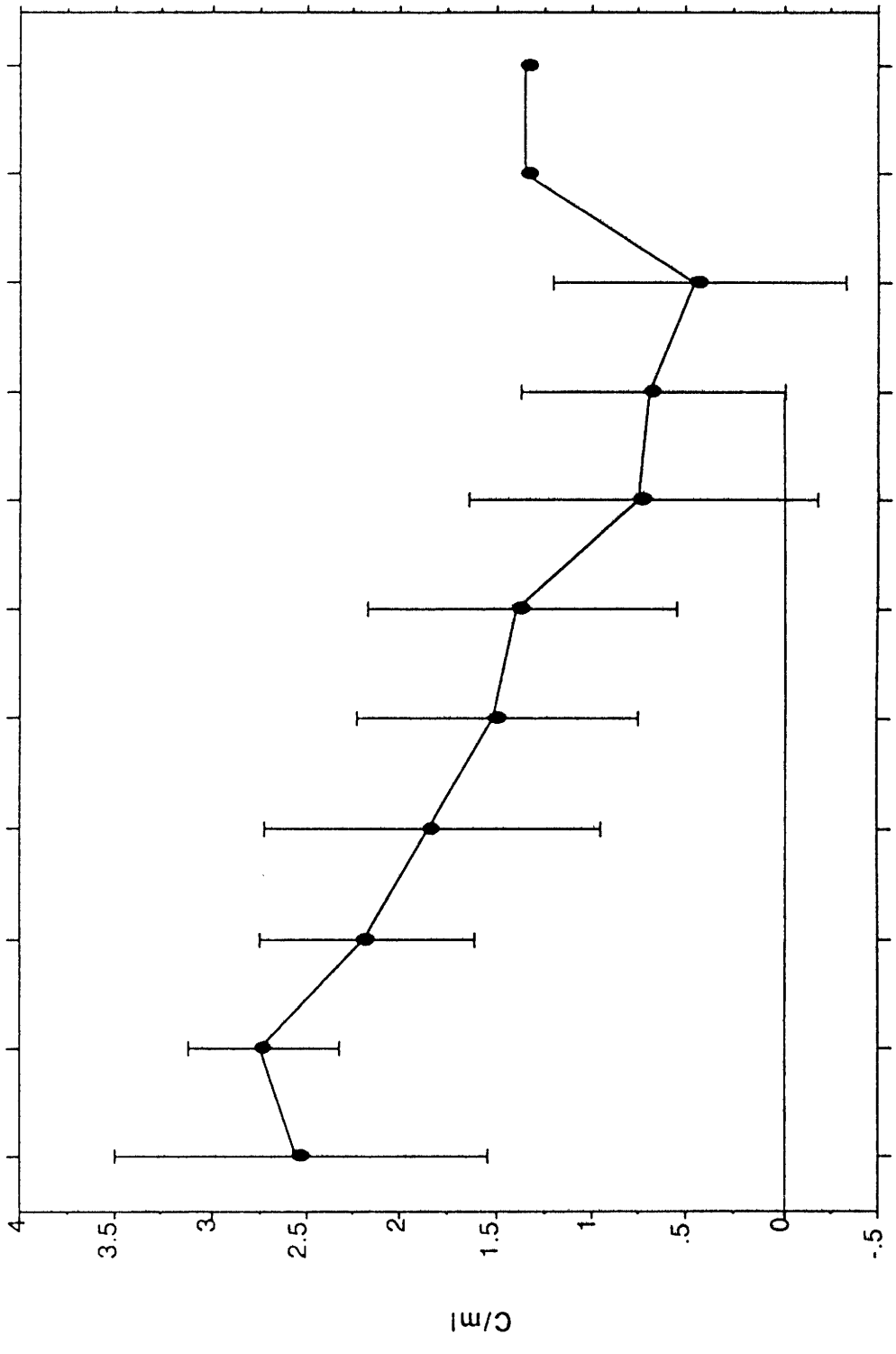


fig. 10 MEAN CELLULARITY IN LTBMC FROM PATIENTS AFTER ABMT

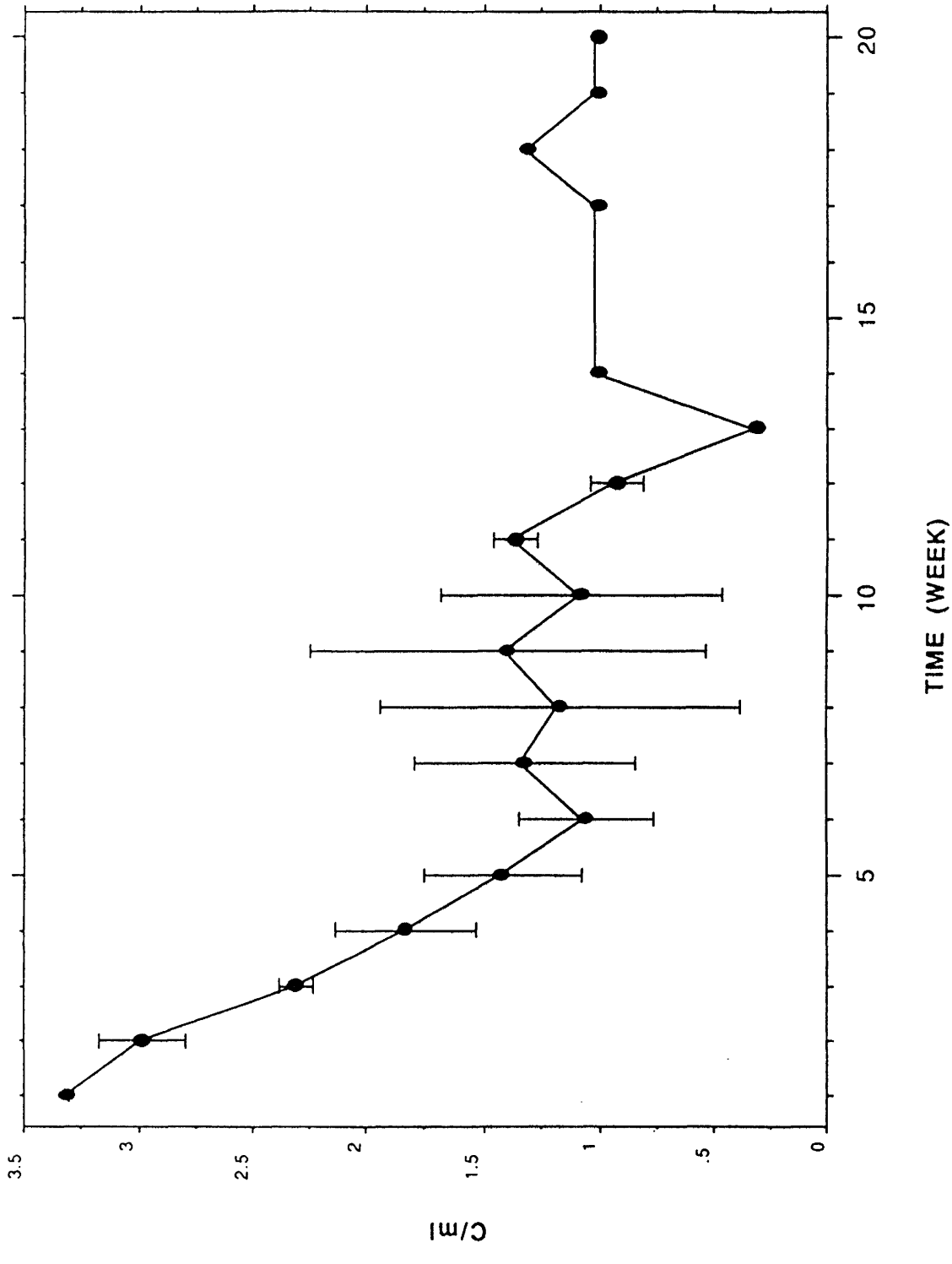


fig. 11 MEAN CELLULARITY IN LTBMC FROM PATIENTS AFTER ABMT AND GM-CSF

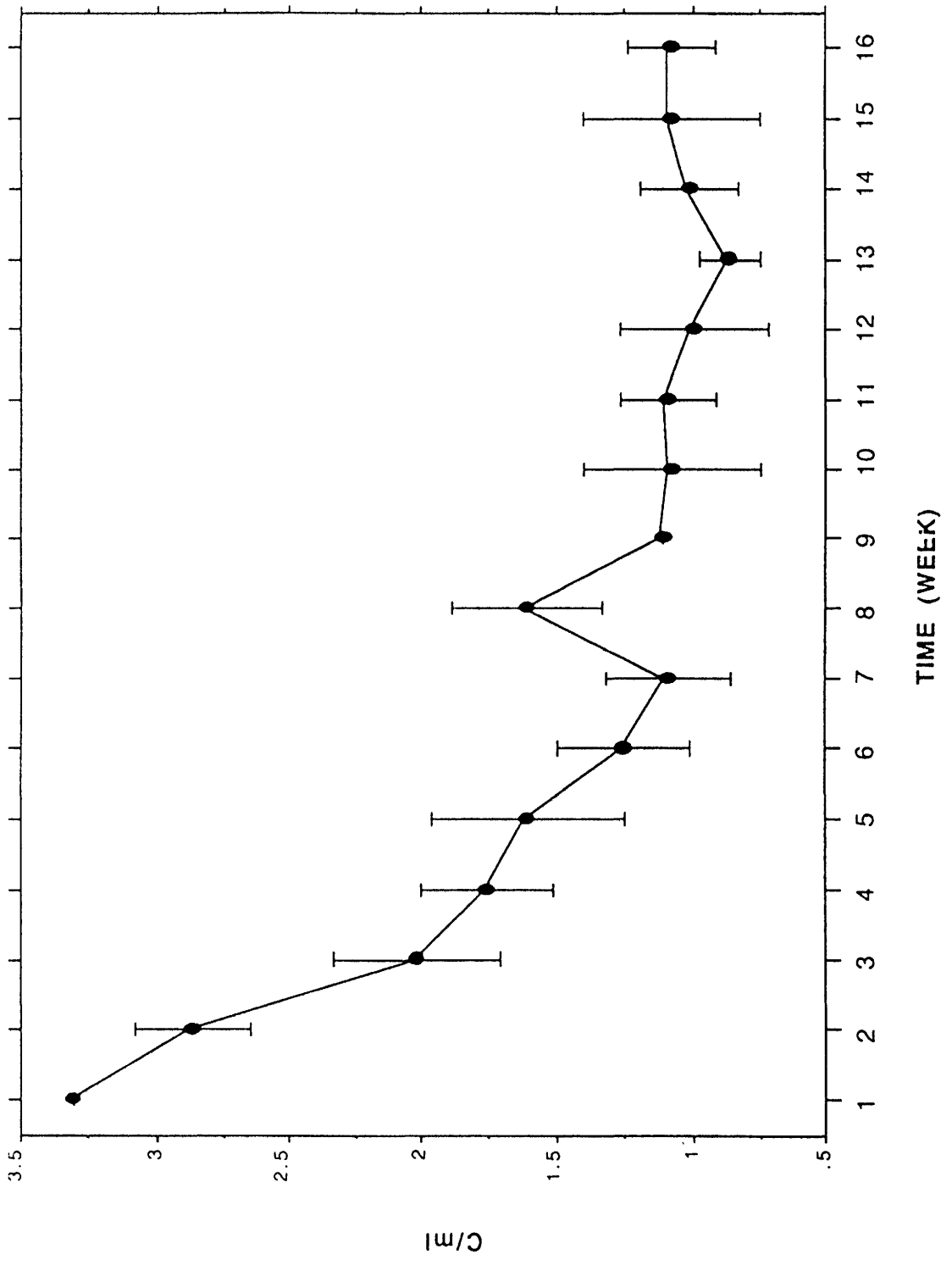


fig. 12 MEAN CLONOGENICITY IN LTBMC FROM PATIENTS AFTER ABMT

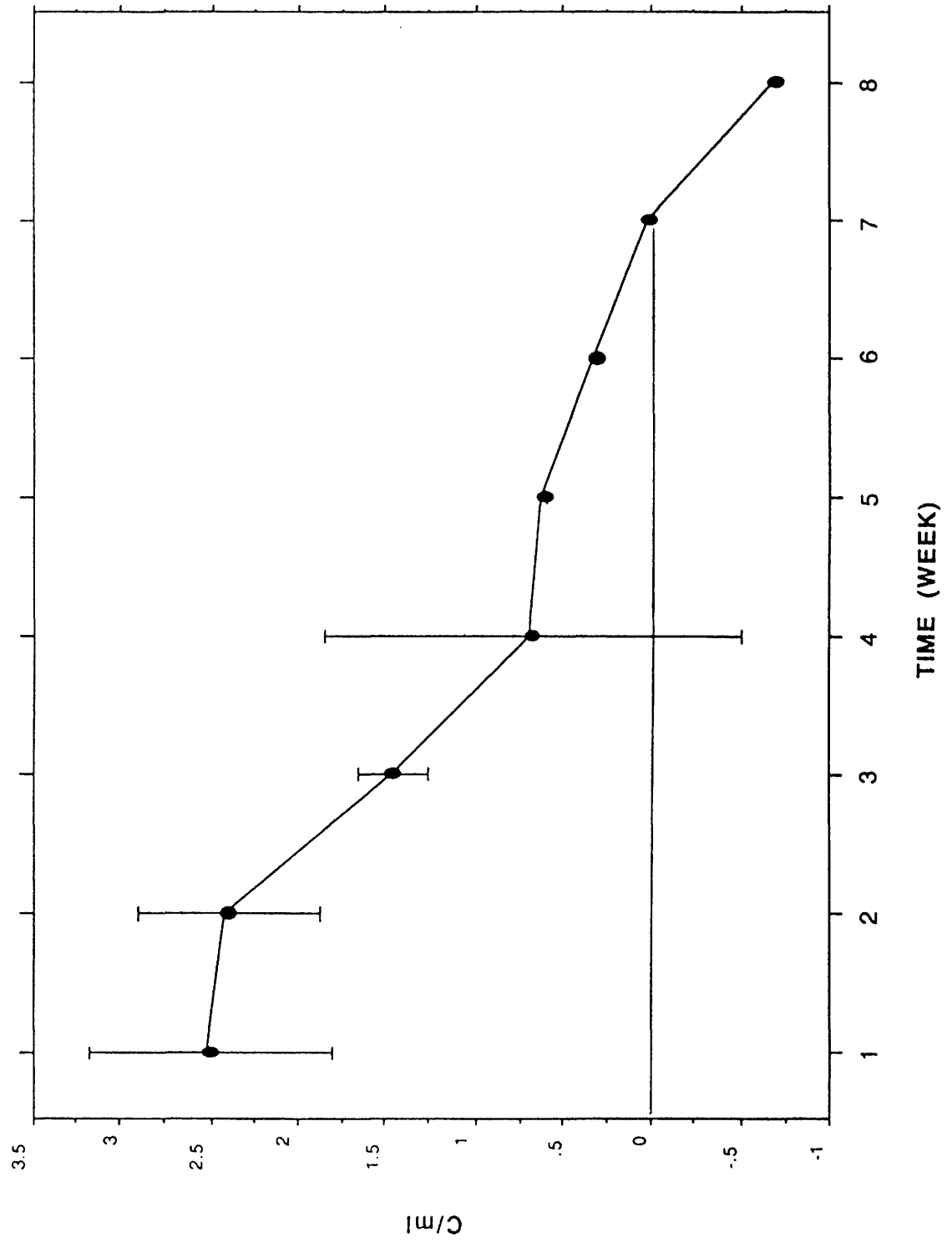


fig. 13 MEAN CLONOGENICITY IN LTBMC FROM PATIENTS AFTER ABMT AND GM-CSF

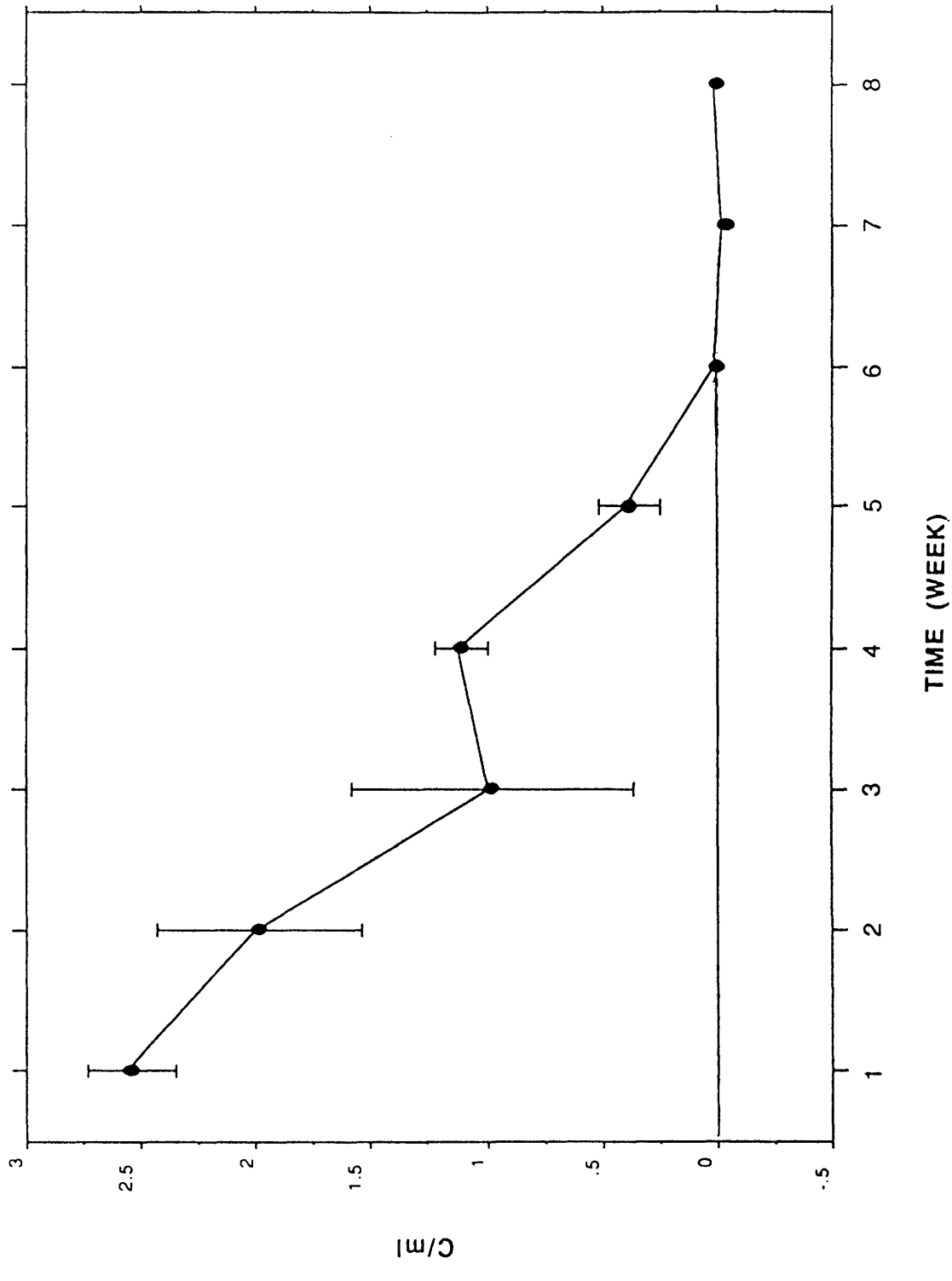


fig. 14 LATE SURVEY OF CLONOGENICITY IN LTBMC AFTER ABMT WITHOUT GM-CSF

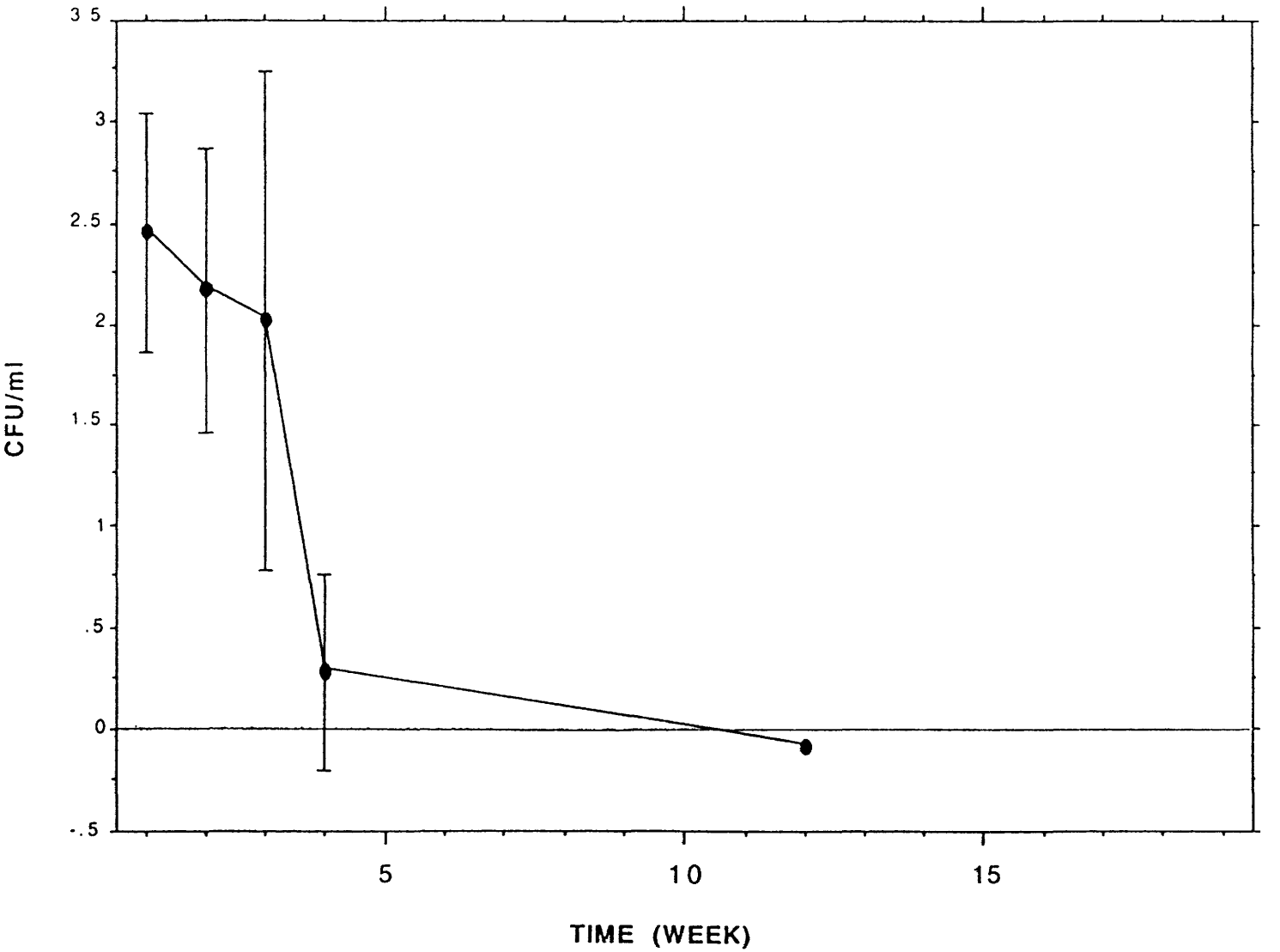


fig. 15 LATE SURVEY OF CELLULARITY IN LTBMC AFTER ABMT WITH GM-CSF

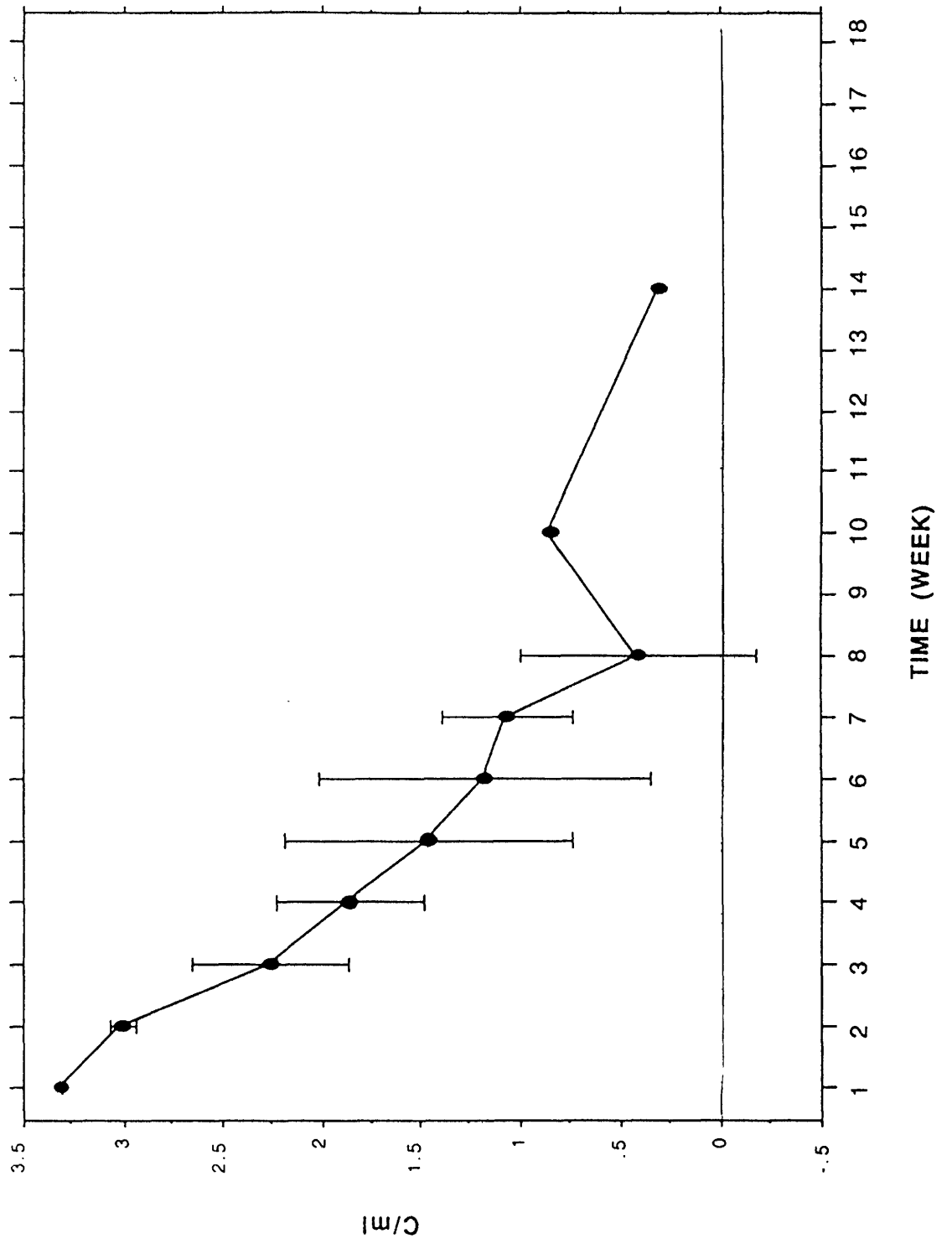


fig. 16 LATE SURVEY OF CELLULARITY IN LTBMC AFTER ABMT WITHOUT GM-CSF

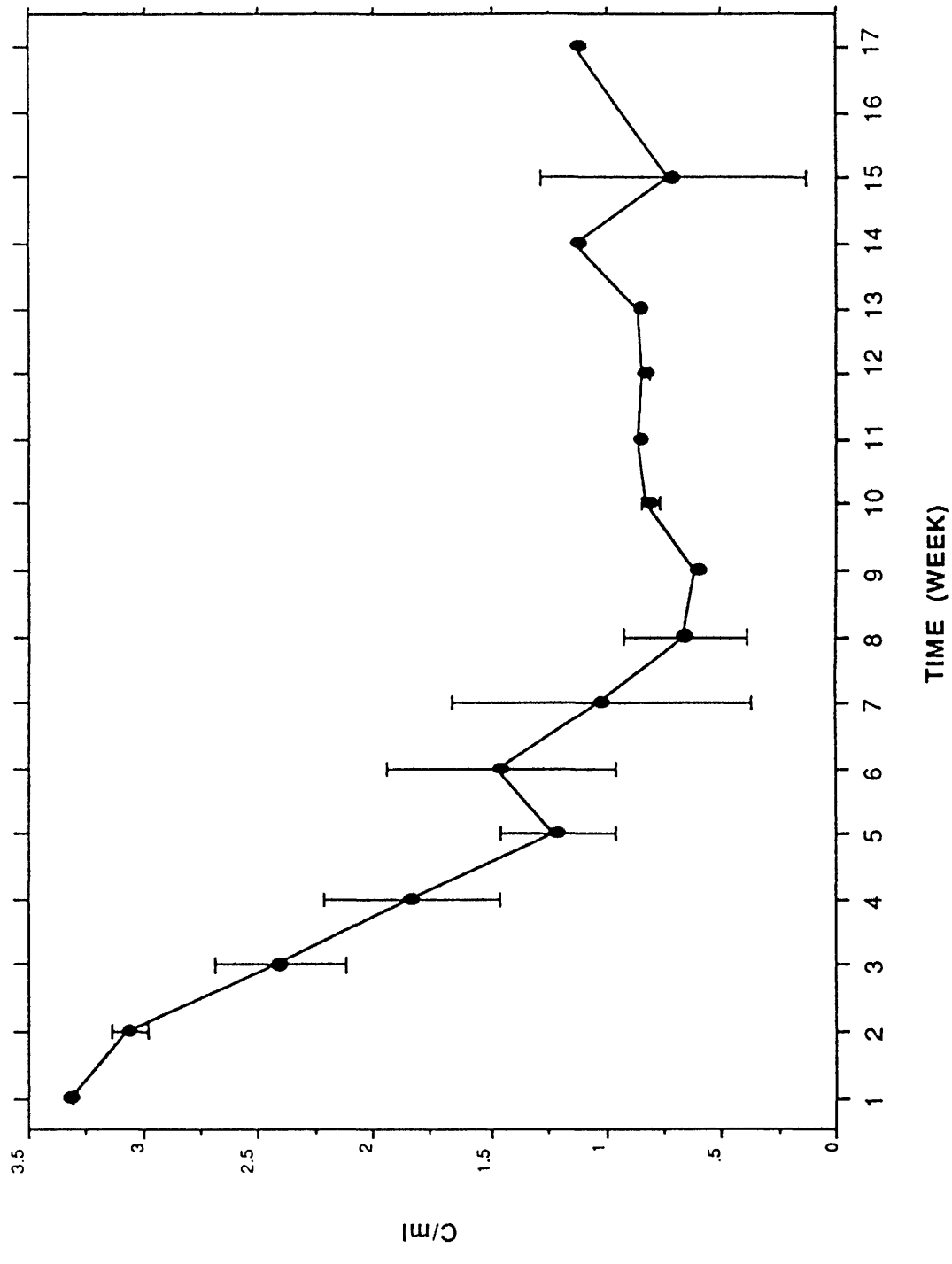


fig. 17 LATE SURVEY OF CLONOGENICITY AFTER ABMT WITH GM-CSF

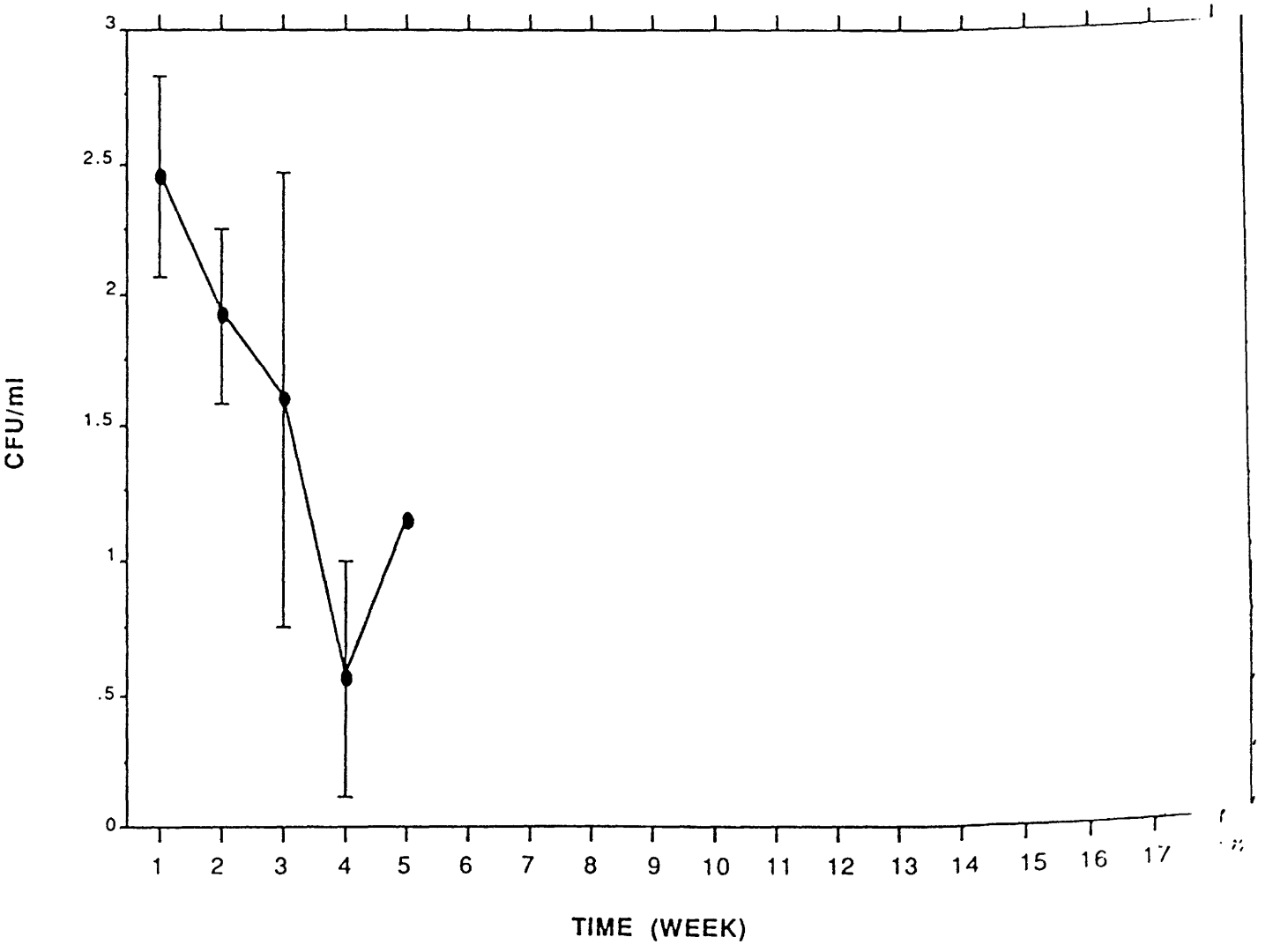


fig. 18 MEAN LEUKOCYTES COUNTS IN GM-CSF TREATMENT

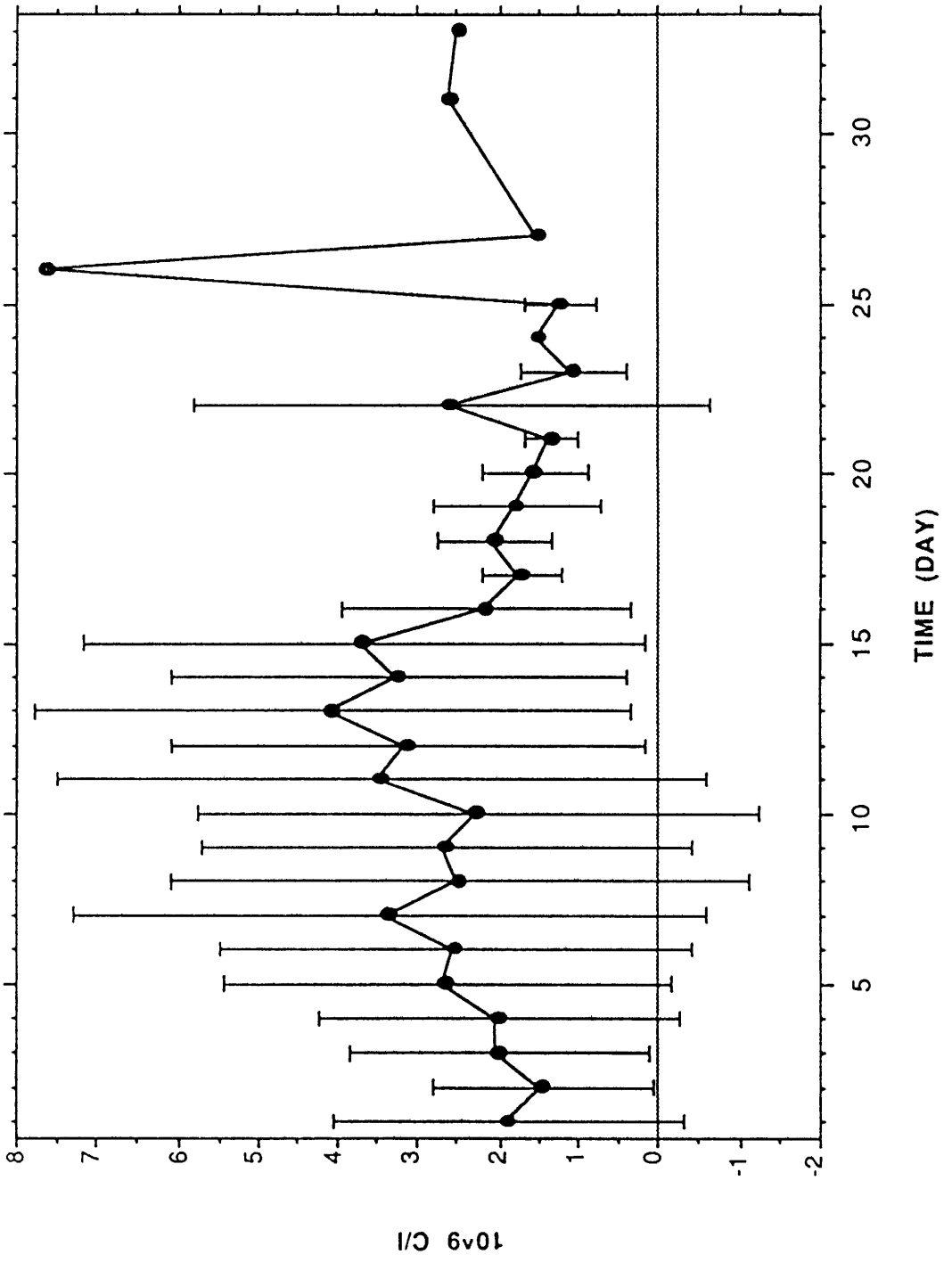


fig. 19 MEAN ERYTHROCYTES COUNTS IN GM-CSF TREATMENT

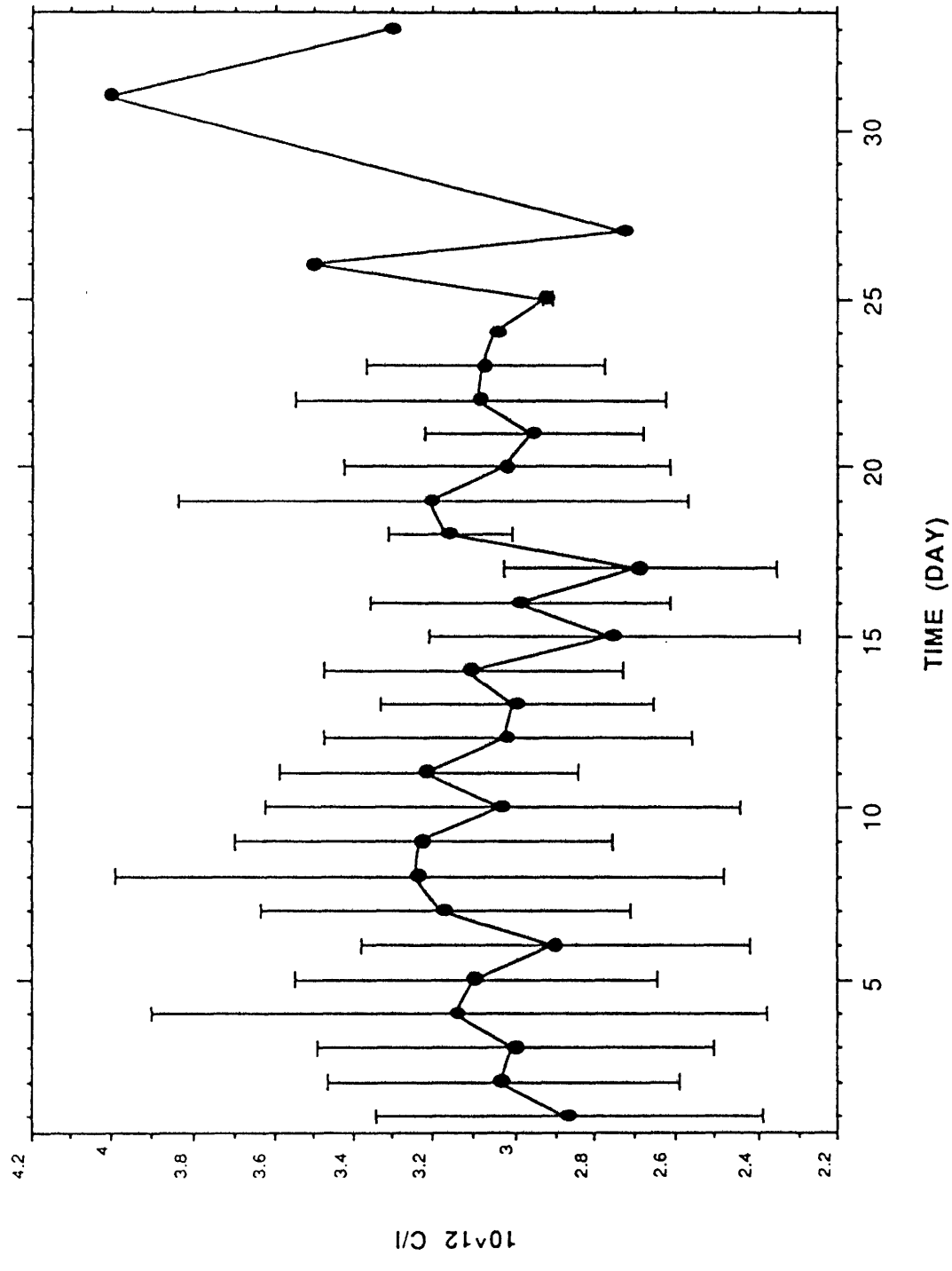


fig. 20 MEAN PLATELETS COUNTS DURING GM-CSF TREATMENT

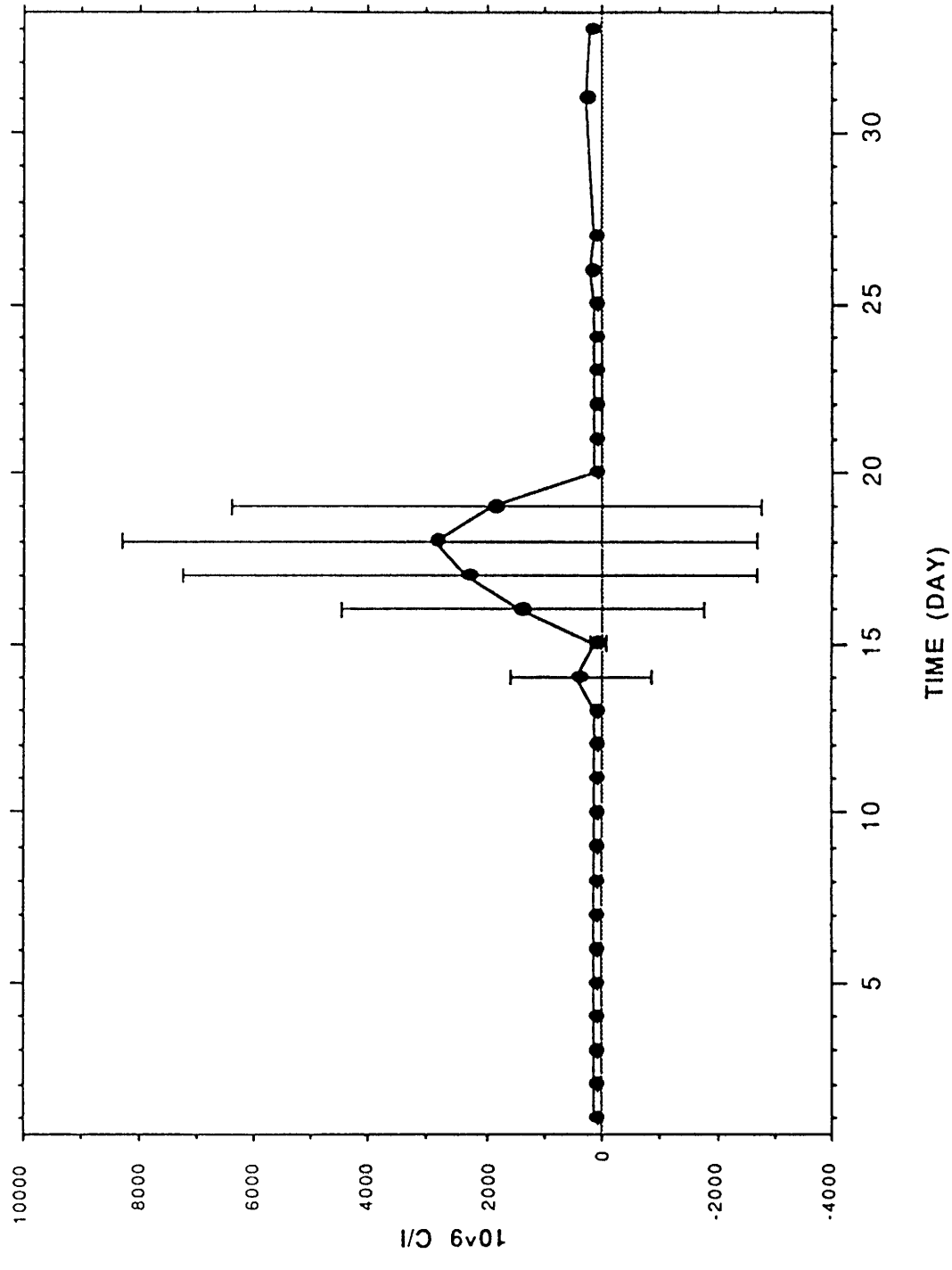


fig. 21 MEAN HEMOGLOBIN LEVEL DURING GM-CSF TREATMENT

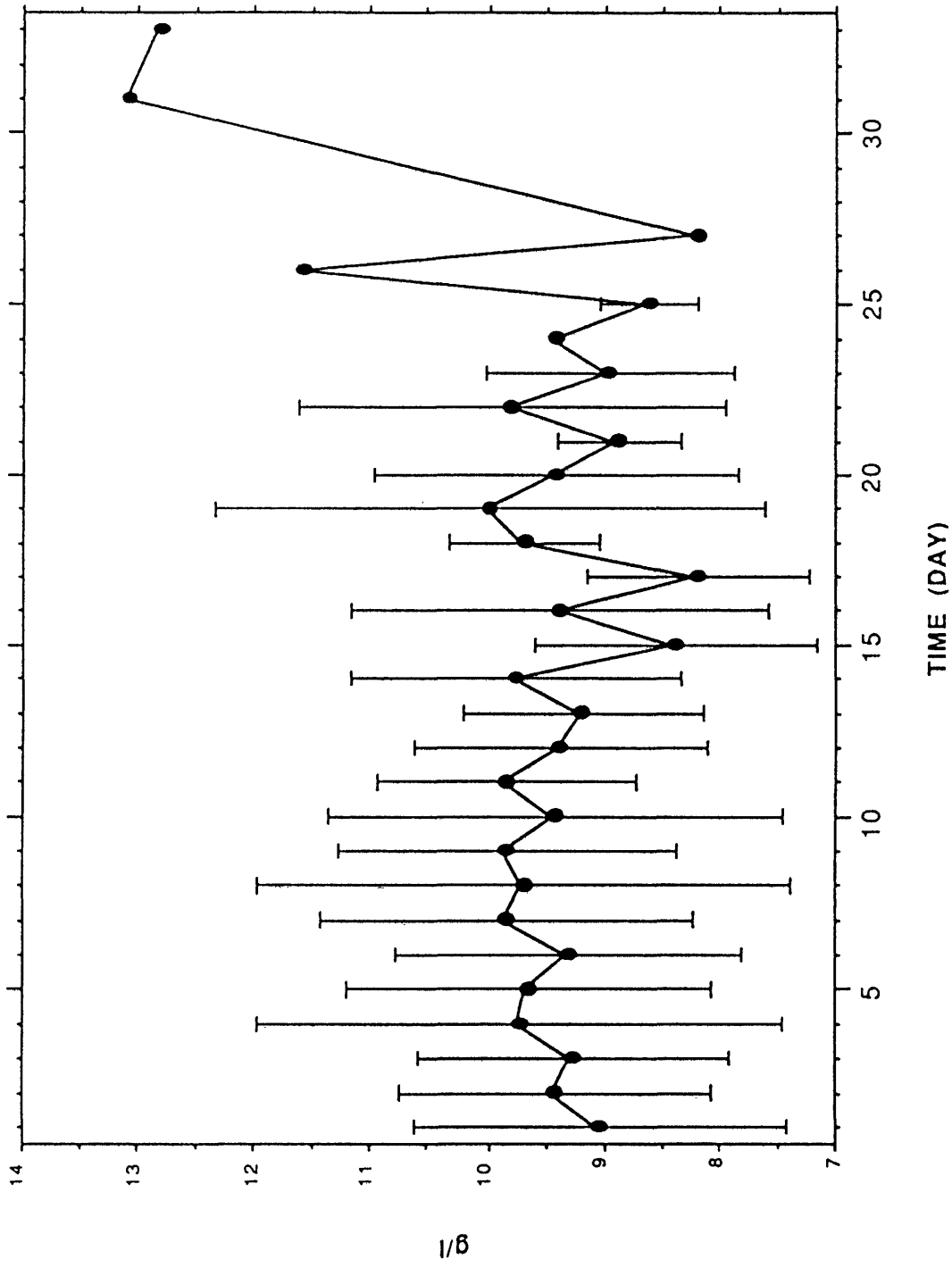


fig. 22 MEAN HEMATOCRIT DURING GM-CSF TREATMENT

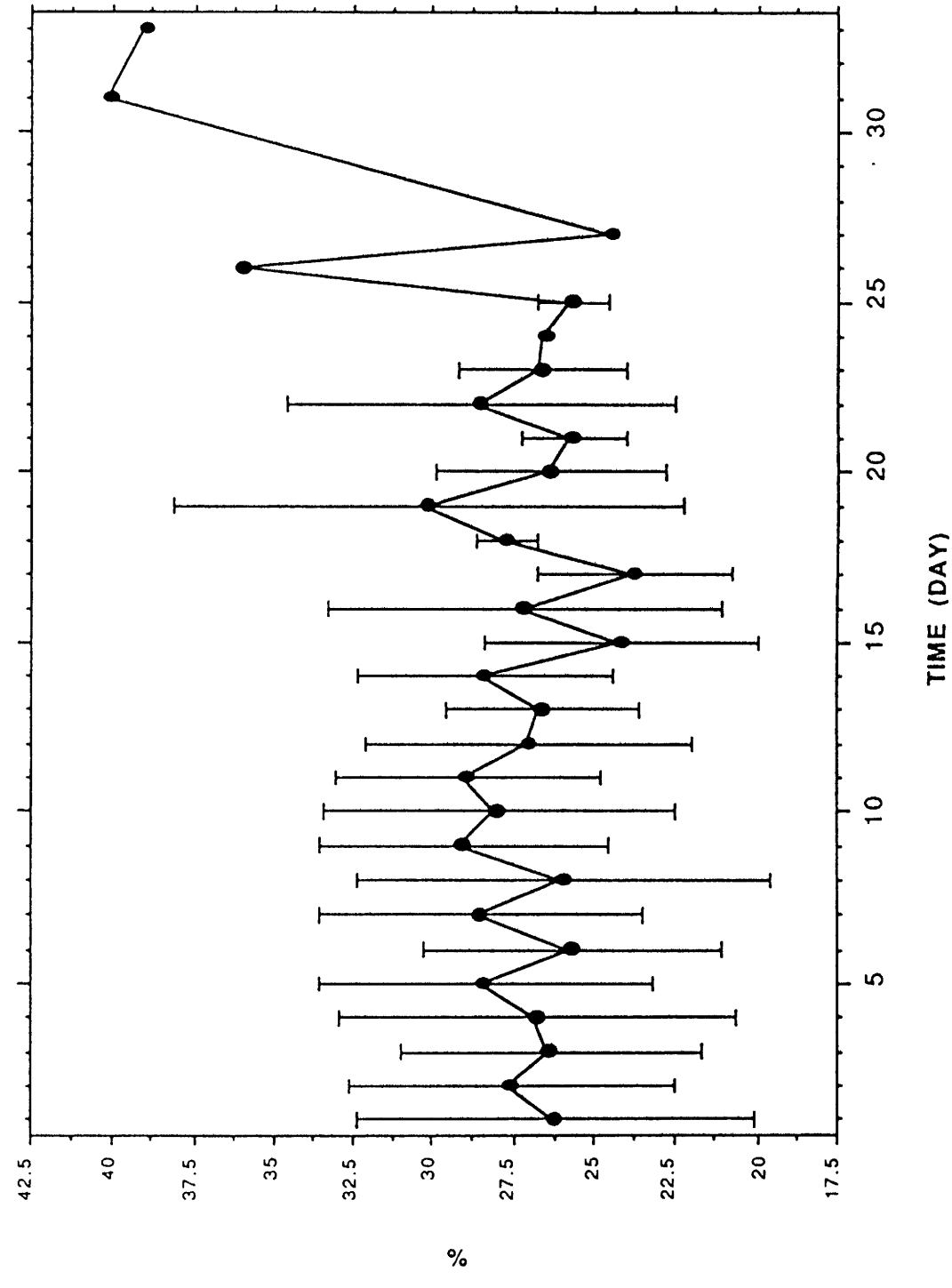


fig. 23a LEUKOCYTES COUNTS IN GM-CSF TREATMENTS OF ONE PATIENT

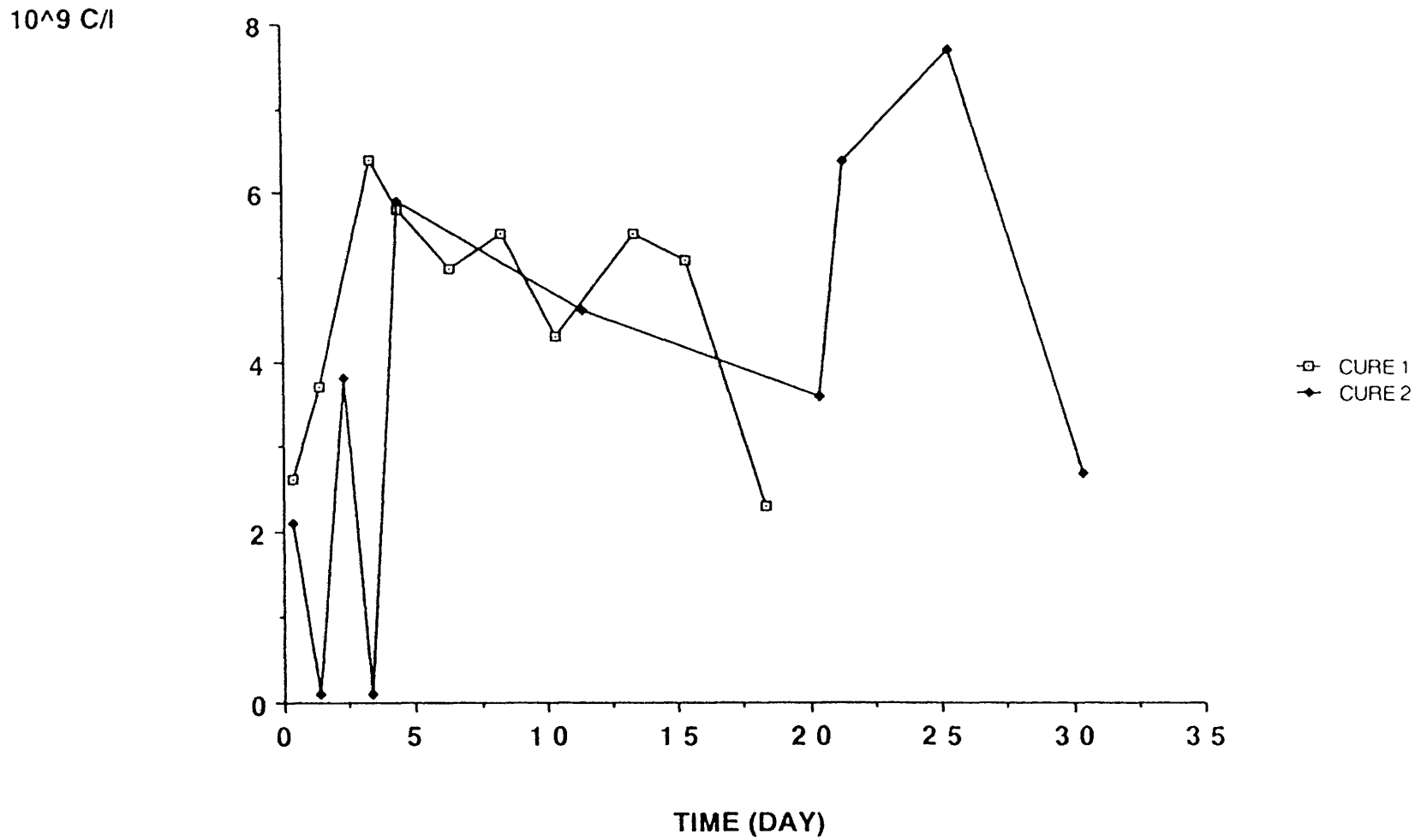


fig. 23b LEUKOCYTES COUNTS IN GM-CSF TREATMENTS OF ONE PATIENT

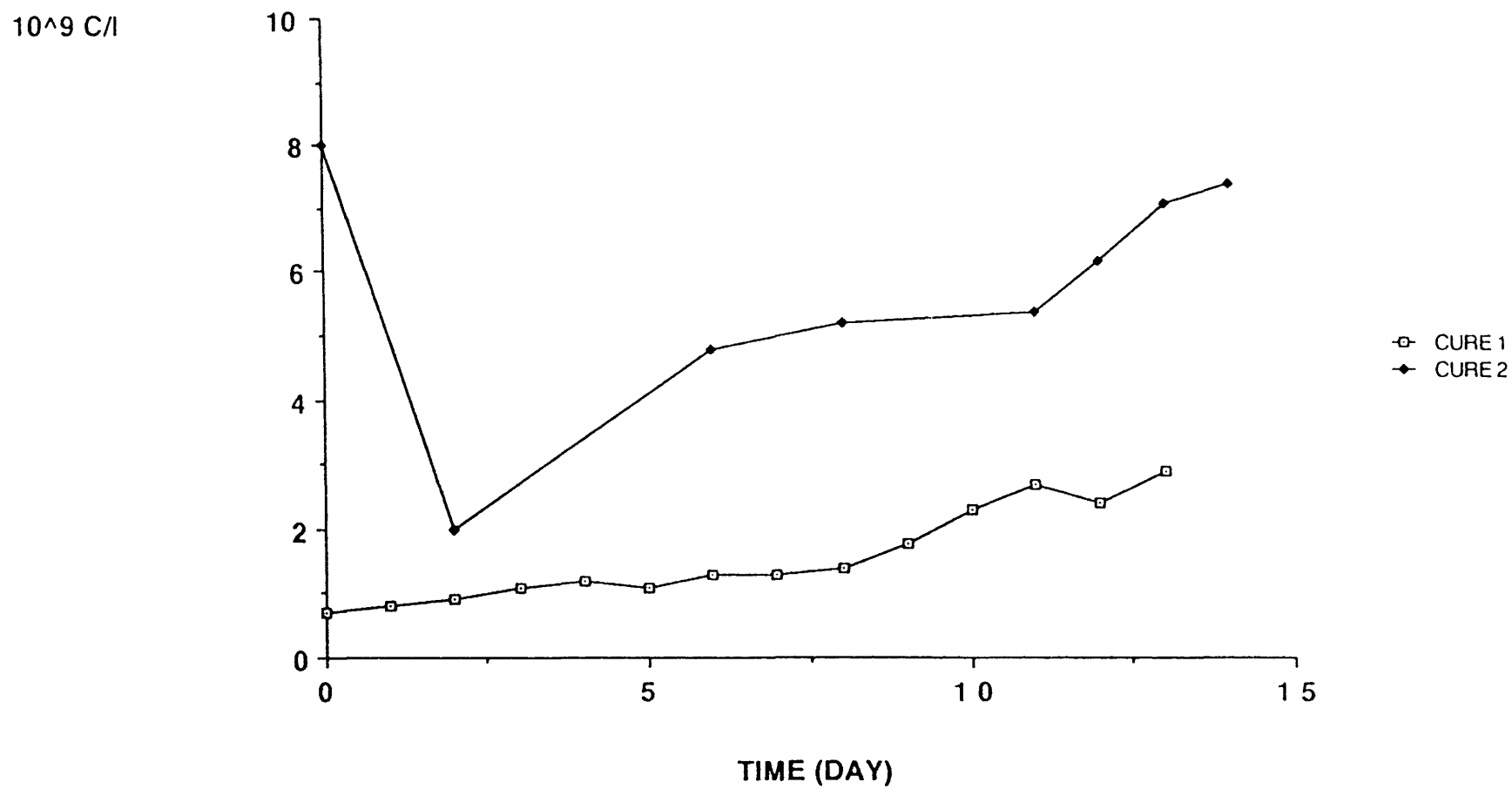


fig. 23c LEUCOCYTES COUNTS IN GM-CSF TREATMENTS OF ONE PATIENT

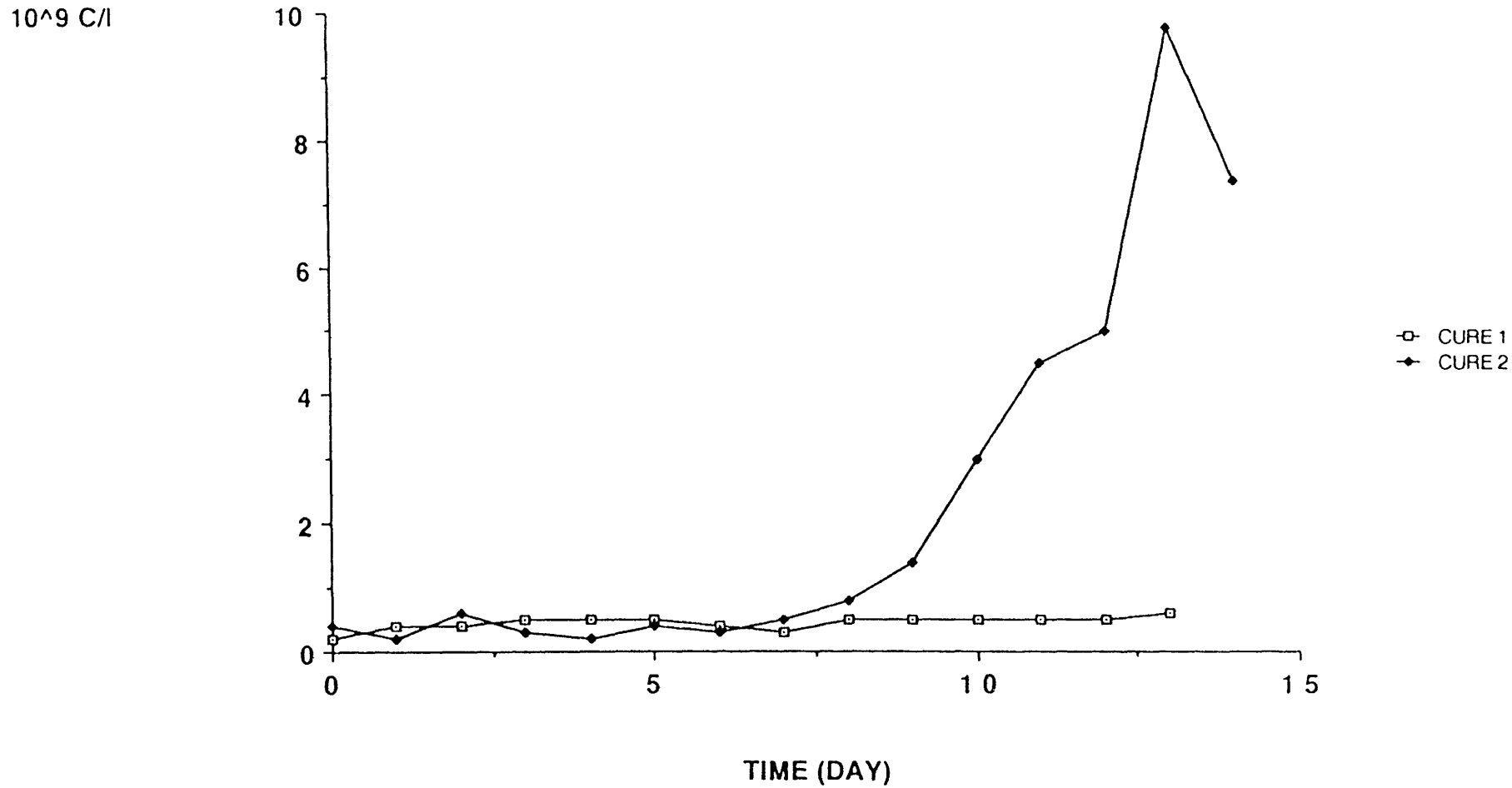


fig. 23d LEUKOCYTES COUNTS IN GM-CSF TREATMENTS OF ONE PATIENT

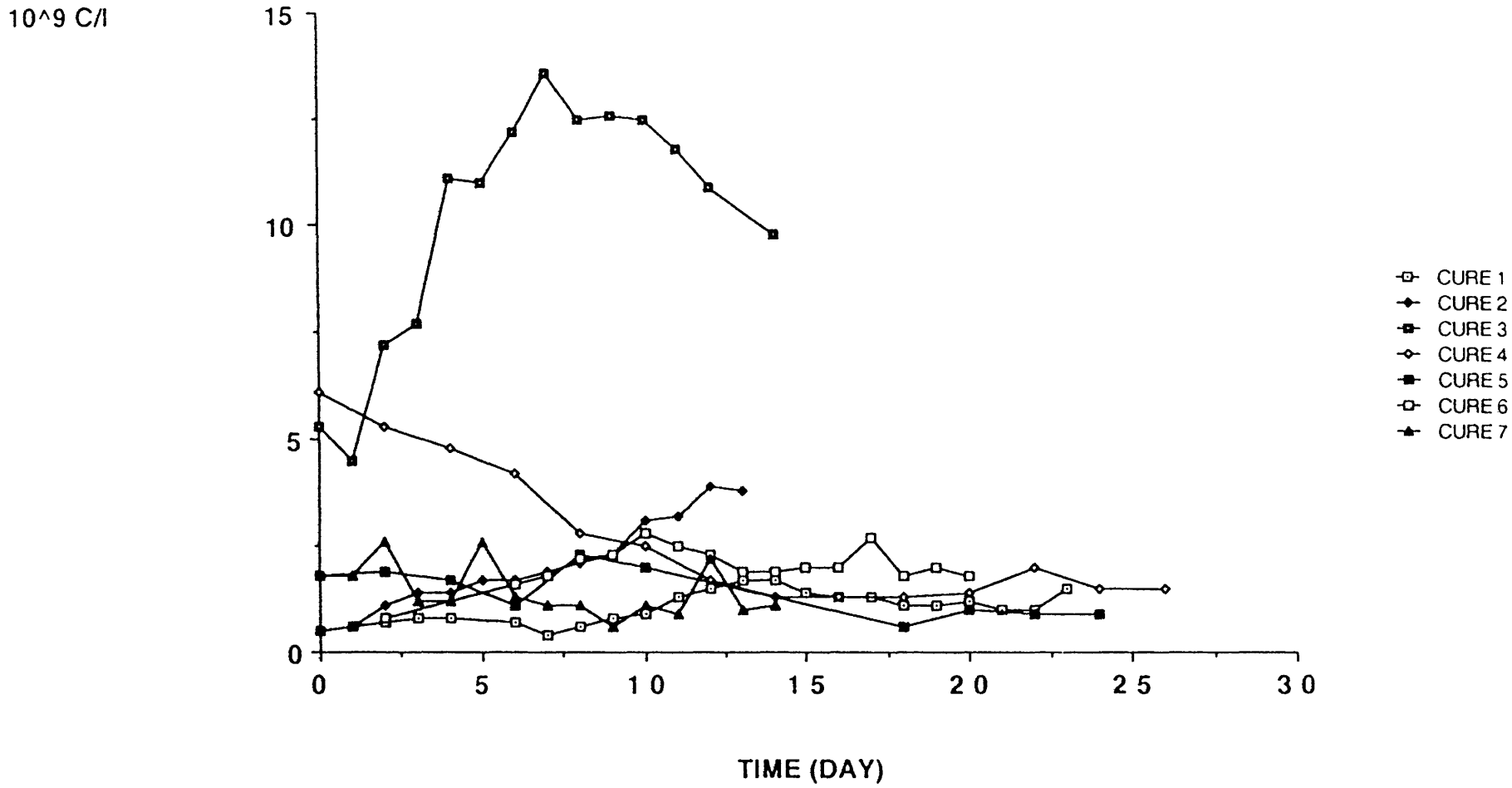
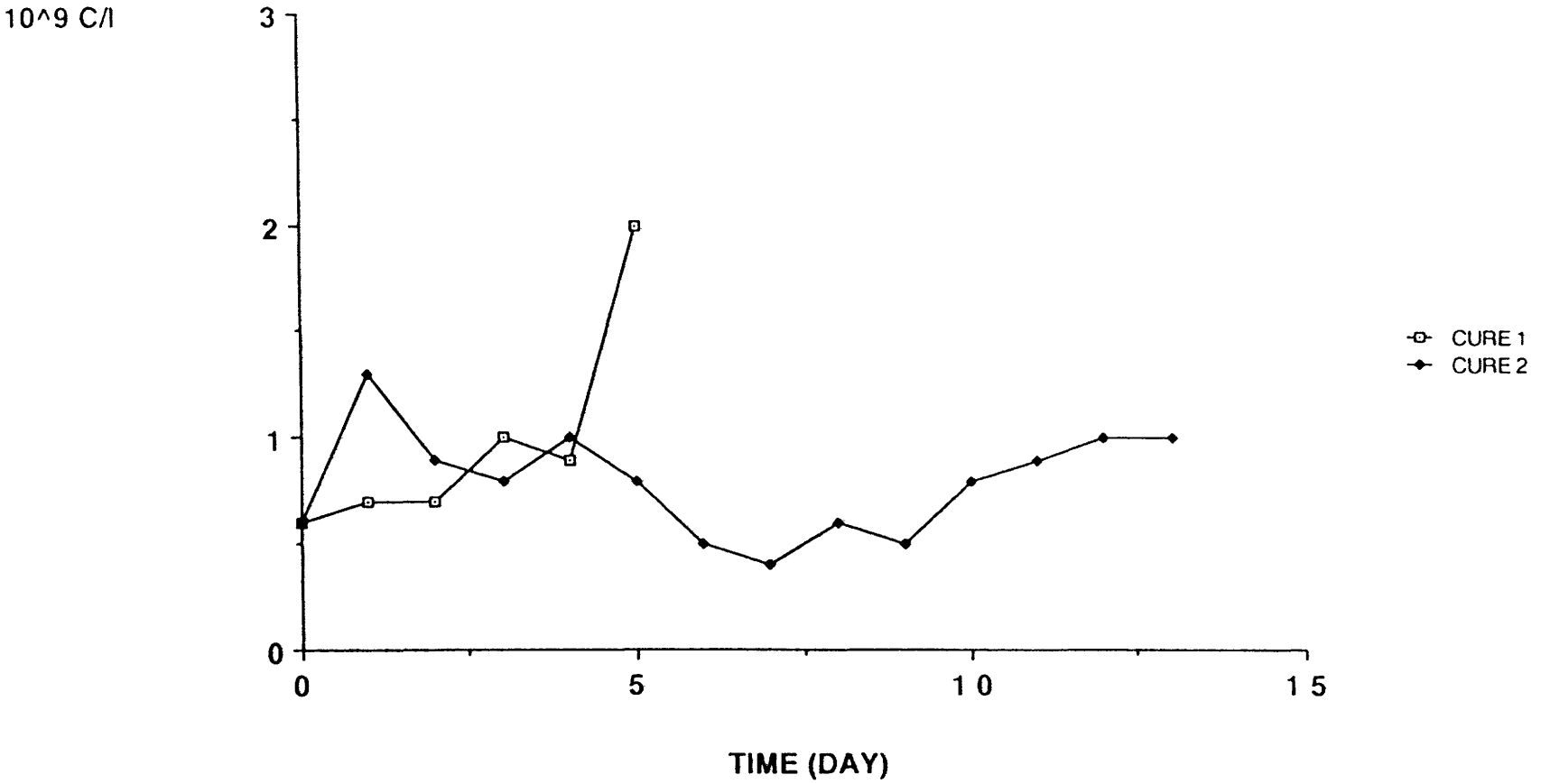


fig. 23e LEUKOCYTES COUNTS IN GM-CSF TREATMENTS OF ONE PATIENT



European Communities — Commission

EUR 12558 — Treatment and biological dosimetry of exposed persons Post-Chernobyl action

Edited by: *K. Chadwick, G. Gerber*

Luxembourg: Office for Official Publications of the European Communities

1991 — XXV, 205 pp., num. tab., fig. — 21.0 × 29.7 cm

Radiation protection series

ISBN 92-826-3064-1

Catalogue number: CD-NA-12558-EN-C

Price (excluding VAT) in Luxembourg: ECU 18.75

The Chernobyl accident, which occurred on 26 April 1986, presented major challenges to the European Community with respect to the practical and regulatory aspects of radiation protection, public information, trade, particularly in food, and international politics. The Chernobyl accident was also a major challenge to the international scientific community which had to evaluate rapidly the radiological consequences of the accident and advise on the introduction of any countermeasures. Prior to the accident at Chernobyl, countermeasures to reduce the consequences of radioactive contamination had been conceived largely in the context of relatively small accidental releases and for application over relatively small areas. Less consideration had been given to the practical implications of applying such measures in case of a large source term and a spread over a very large area.

Venta y suscripciones • Salg og abonnement • Verkauf und Abonnement • Πωλήσεις και συνδρομές
Sales and subscriptions • Vente et abonnements • Vendita e abbonamenti
Verkoop en abonnementen • Venda e assinaturas

BELGIQUE / BELGIË

Moniteur belge / Belgisch Staatsblad
Rue de Louvain 42 / Leuvenseweg 42
1000 Bruxelles / 1000 Brussel
Tél. (02) 512 00 26
Fax 511 01 84
CCP / Postrekening 000-2005502-27

Autres distributeurs /
Overige verkooppunten

Librairie européenne/ Europese Boekhandel
Avenue Albert Jonnart 50 /
Albert Jonnartlaan 50
1200 Bruxelles / 1200 Brussel
Tél. (02) 734 02 81
Fax 735 08 60

Jean De Lannoy
Avenue du Roi 202 /Koningslaan 202
1060 Bruxelles / 1060 Brussel
Tél. (02) 538 51 69
Télex 63220 UNBOOK B
Fax (02) 538 08 41

CREDOC
Rue de la Montagne 34 / Bergstraat 34
Bte 11 / Bus 11
1000 Bruxelles / 1000 Brussel

DANMARK

J. H. Schultz Information A/S
EF-Publikationer
Ottiliavej 18
2500 Valby
Tlf. 36 44 22 66
Fax 36 44 01 41
Girokonto 6 00 08 86

BR DEUTSCHLAND

Bundesanzeiger Verlag
Breite Straße
Postfach 10 80 06
5000 Köln 1
Tel. (02 21) 20 29-0
Telex ANZEIGER BONN 8 882 595
Fax 20 29 278

GREECE

G.C. Eleftheroudakis SA
International Bookstore
Nikis Street 4
10563 Athens
Tel. (01) 322 63 23
Telex 219410 ELEF
Fax 323 98 21

ESPAÑA

Boletín Oficial del Estado
Trafalgar, 27
28010 Madrid
Tel. (91) 44 82 135

Mundi-Prensa Libros, S.A.
Castelló, 37
28001 Madrid
Tel. (91) 431 33 99 (Libros)
431 32 22 (Suscripciones)
435 36 37 (Dirección)
Télex 49370-MPLI-E
Fax (91) 575 39 98

Sucursal:

Librería Internacional AEDOS
Consejo de Ciento, 391
08009 Barcelona
Tel. (93) 301 86 15
Fax (93) 317 01 41

Llibreria de la Generalitat de Catalunya
Rambla dels Estudis, 118 (Palau Moja)
08002 Barcelona
Tel. (93) 302 68 35
302 64 62
Fax (93) 302 12 99

FRANCE

Journal officiel
Service des publications des Communautés européennes
26, rue Desaix
75727 Paris Cedex 15
Tél. (1) 40 58 75 00
Fax (1) 40 58 75 74

IRELAND

Government Publications Sales Office
Sun Alliance House
Molesworth Street
Dublin 2
Tel. (1) 71 03 09

or by post

Government Stationery Office
EEC Section
6th floor
Bishop Street
Dublin 8
Tel. (1) 78 16 66
Fax (1) 78 06 45

ITALIA

Licosa Spa
Via Benedetto Fortini, 120/10
Casella postale 552
50125 Firenze
Tel. (055) 64 54 15
Fax 64 12 57
Telex 570466 LICOSA I
CCP 343 509

Subagenti:

Libreria scientifica
Lucio de Biasio - AEIOU
Via Meravigli, 16
20123 Milano
Tel. (02) 80 76 79

Herder Editrice e Libreria
Piazza Montecitorio, 117-120
00186 Roma
Tel. (06) 679 46 28/679 53 04

Libreria giuridica
Via XII Ottobre, 172/R
16121 Genova
Tel. (010) 59 56 93

GRAND-DUCHÉ DE LUXEMBOURG

Messageries Paul Kraus
11, rue Christophe Plantin
2339 Luxembourg
Tél. 499 88 88
Télex 2515
Fax 499 88 84 44
CCP 49242-63

NEDERLAND

SDU Overheidsinformatie
Externe Fondsen
Postbus 20014
2500 EA 's-Gravenhage
Tel. (070) 37 89 911
Fax (070) 34 75 778

PORTUGAL

Imprensa Nacional
Casa da Moeda, EP
Rua D. Francisco Manuel de Melo, 5
1092 Lisboa Codex
Tel. (01) 69 34 14

Distribuidora de Livros Bertrand, Ld.ª
Grupo Bertrand, SA
Rua das Terras dos Vales, 4-A
Apartado 37
2700 Amadora Codex
Tel. (01) 49 59 050
Telex 15798 BERDIS
Fax 49 60 255

UNITED KINGDOM

HMSO Books (PC 16)
HMSO Publications Centre
51 Nine Elms Lane
London SW8 5DR
Tel. (071) 873 2000
Fax GP3 873 8463
Telex 29 71 138

ÖSTERREICH

Manz'sche Verlags- und Universitätsbuchhandlung
Kohlmarkt 16
1014 Wien
Tel. (0222) 531 61-0
Telex 11 25 00 BOX A
Fax (0222) 531 61-81

SUOMI

Akateeminen Kirjakauppa
Keskuskatu 1
PO Box 128
00101 Helsinki
Tel. (0) 121 41
Fax (0) 121 44 41

NORGE

Narvesen Information center
Bertrand Narvesens vei 2
PO Box 6125 Etterstad
0602 Oslo 6
Tel. (2) 57 33 00
Telex 79668 NIC N
Fax (2) 68 19 01

SVERIGE

BTJ
Box 200
22100 Lund
Tel. (046) 18 00 00
Fax (046) 18 01 25

SCHWEIZ / SUISSE / SVIZZERA

OSEC
Stampfenbachstraße 85
8035 Zürich
Tel. (01) 365 54 49
Fax (01) 365 54 11

CESKOSLOVENSKO

NIS
Havelkova 22
13000 Praha 3
Tel. (02) 235 84 46
Fax 42-2-264775

MAGYARORSZÁG

Agroinform
Budapest I. Kir.
Attila út 93
1012 Budapest
Tel. (1) 56 82 11
Telex (22) 4717 AGINF H-61

POLAND

Business Foundation
ul. Krucza 38/42
00-512 Warszawa
Tel. (22) 21 99 93, 628-28-82
International Fax&Phone
(0-39) 12-00-77

YUGOSLAVIA

Privredni Vjesnik
Bulevar Lenjina 171/XIV
11070 Beograd
Tel. (11) 123 23 40

CYPRUS

Cyprus Chamber of Commerce and Industry
Chamber Building
38 Grivas Dhigenis Ave
3 Deligiorgis Street
PO Box 1455
Nicosia
Tel. (2) 449500/462312
Fax (2) 458630

TÜRKIYE

Pres Gazete Kitap Dergi Pazarlama Dağitim Ticaret ve sanayi AŞ
Narlibahçe Sokak N. 15
Istanbul-Cağaloğlu
Tel. (1) 520 92 96 - 528 55 66
Fax 520 64 57
Telex 23822 DSVO-TR

AUTRES PAYS
OTHER COUNTRIES
ANDERE LÄNDER

Office des publications officielles des Communautés européennes
2, rue Mercier
2985 Luxembourg
Tél. 49 92 81
Télex PUBOF LU 1324 b
Fax 48 85 73
CC bancaire BIL 8-109/6003/700

CANADA

Renouf Publishing Co. Ltd
Mail orders — Head Office:
1294 Algoma Road
Ottawa, Ontario K1B 3W8
Tel. (613) 741 43 33
Fax (613) 741 54 39
Telex 0534783

Ottawa Store:
61 Sparks Street
Tel. (613) 238 89 85

Toronto Store:
211 Yonge Street
Tel. (416) 363 31 71

UNITED STATES OF AMERICA

UNIPUB
4611-F Assembly Drive
Lanham, MD 20706-4391
Tel. Toll Free (800) 274 4888
Fax (301) 459 0056

AUSTRALIA

Hunter Publications
58A Gipps Street
Collingwood
Victoria 3066

JAPAN

Kinokuniya Company Ltd
17-7 Shinjuku 3-Chome
Shinjuku-ku
Tokyo 160-91
Tel. (03) 3439-0121

Journal Department
PO Box 55 Chitose
Tokyo 156
Tel. (03) 3439-0124

CD-NA-12558-EN-C

NOTICE TO THE READER

All scientific and technical reports published by the Commission of the European Communities are announced in the monthly periodical **'euro abstracts'**. For subscription (1 year: ECU 92) please write to the address below.

Price (excluding VAT) in Luxembourg: ECU 18.75

ISBN 92-826-3064-1

OFFICE FOR OFFICIAL PUBLICATIONS
OF THE EUROPEAN COMMUNITIES

L-2985 Luxembourg