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General information

HEALTH RESEARCH WITH DEVELOPING COUNTRIES

Volume 3
PARASITOLOGY

OVERVIEW OF EC SUPPORTED JOINT RESEARCH PROJECTS

RTD INTERNATIONAL COOPERATION

EUROPEAN COMMISSION

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European Commission

Directorate-General XII:
Science, Research and Development

HEALTH RESEARCH WITH DEVELOPING COUNTRIES

Volume 3

PARASITOLOGY

MALARIA
SCHISTOSOMIASIS
TRYPANOSOMATIDAE
FILARIASIS
OTHER PARASITES

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Foreword

Health research with developing countries has been part of the European Commission's research agenda under its regular research budget for almost 15 years.

It is currently part of the specific RTD Programme in the field of Cooperation with Third Countries and International Organizations (INCO): activity II of the Framework Programme IV (1994-1998) (see annex).

The selection of research areas is based on existing and newly identified health needs in developing countries and on the capacity to cope with these needs through research.

This document presents the "parasitology" component of the health sector under the INCO-developing countries programme (INCO-DC) and its predecessor STD-3 (1991-1994). It combines summaries of completed contracts for STD3 and a catalogue of ongoing and new contracts of STD3 and INCO-DC, 1st and 2nd call. Other health research areas including work on the tools for prevention and control of viral, bacterial and non-communicable diseases and health systems research are presented in other volumes.

The parasitic diseases remain a major health problem in many developing countries. While there have been notable successes in the fight against parasitic diseases in recent times, for example Chagas' disease vector control in Latin America and Onchocerciasis control in West Africa, the general situation has not improved and in some cases has deteriorated. Science has offered the technical ability to have a major input to a wider variety of health needs in developing countries and the parasitology research reported here has focused on attempts to better understand the biology of disease and to provide the tools for successful interventions including vaccines, drugs and diagnostic products.

Through the STD and INCO-DC programmes particular emphasis is given to the process of partnership-building in science. Capacity and capability development for all teams involved (in developing countries as well as in the EU) is the key to this process.

Scientific cooperation being an integral part of the EU research policy is therefore an essential instrument in support of the development policy of the EU.

The specific objectives and the operational modalities are dealt with in annex 1 and in the information packages which are produced to accompany each 'call for proposals'.

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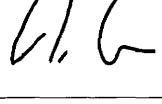


R. Gerold
Director

The INCO-DC Workprogramme:
Field of Parasitology: part of health related research

Research on the tools for prevention and the fight against the predominant diseases

Vaccines

Vaccines are cost-effective tools for disease control. Research on vaccines in the programme should be concentrated on selection, evaluation and delivery of antigens, with the objective of designing vaccines applicable for DC's. For vaccines against diseases with a significant impact in Europe, emphasis will be on aspects specific to DC's. With regard to the evaluation of antigens, emphasis will be put on immunological mechanisms underlying experimentally induced protection. Research on the non-targeted effects of vaccines will also be taken into consideration.

Drugs

Emphasis will be put on targeted drug design and targeted drug delivery, based on the fundamental understanding of biological functions of pathogens and of disease mechanisms. Research on drugs, including bioactive natural compounds is eligible when focused on drugs for predominant diseases in DC's when non-toxic efficient treatments are unavailable.

Diagnostic products

This research should make an initial distinction between 1) the development of diagnostic products as research tools, or 2) the development of diagnostic tools for routine health care. Application of high technology approaches to design robust and simple diagnostic tools for routine health care will be given due attention. The relevance for practical case management of the read-out of routine diagnostic tools should be adequately addressed.

For each project concerning the design and the development of vaccines, drugs and/or diagnostics products, the feasibility of introducing affordable products envisaged should be pre-evaluated.

Research on the biology of the diseases

Biology

The understanding of the processes and biological interactions is a source of new techniques and tools for the control of disease. Research on genome structure and regulation of gene expression is eligible if it supports the investigation of defined biological mechanisms aiming at the control of disease

causing pathogens. Metabolic pathways that differ between the human host and the pathogen may be addressed as a source for targeted interventions. Immunological studies should concentrate on the biological function of antigenic molecules and the mechanisms leading to protection against disease. Studies on the genetics of vectors are supported as tools that allow understanding of the physiological processes of the vectors, and vectorial interaction with the pathogenic agent and the host. Studies of intervention based on vectorial biology are eligible provided that monitoring is envisaged on a longer term and on a sustainable basis.

Pre-clinical models

A model may be used for different purposes. Where a model is proposed as a screen in a development process, the relevance of the particular model for human disease has to be established. *In vitro* and *in vivo* models for human disease will be the subject of studies aimed at refinement, replacement and reduction of animal experimentation.

STD-3 (1991-1994)

Under the 3rd Framework Programme, the predecessor of the INCO-DC programme, the STD-3 programme (Life Sciences and Technologies for Developing Countries: 1991-1994, area Health), resulted in 140 health related STD-3 contracts. Of these 69 were on *Parasitology*. 283 scientific teams from 185 institutes, involving 50 countries received European Commission support for this specific subtopic totaling 19.8 Million ECU. This represented about 51.3% of the total health research related budget of this EC RTD programme (Figure).

INCO-DC (1994-1998)

Under the 4th Framework Programme, the INCO-DC programme (sector health) has, as a result of the first and second call in 1995 and in 1996, selected 48 projects related to *Parasitology* involving 52 countries. A budget of 19.18 Million ECU (so far 46.7% of the contribution to the health sector of INCO-DC) has been in support of 177 teams from 153 institutes dealing with this component of health research (Figure).

The evaluation process related to the 3rd Call for Proposals of 1997 has selected another group of 20 *Parasitology* projects with an estimated budget of 8.68 Million ECU, to be operational in 1998.

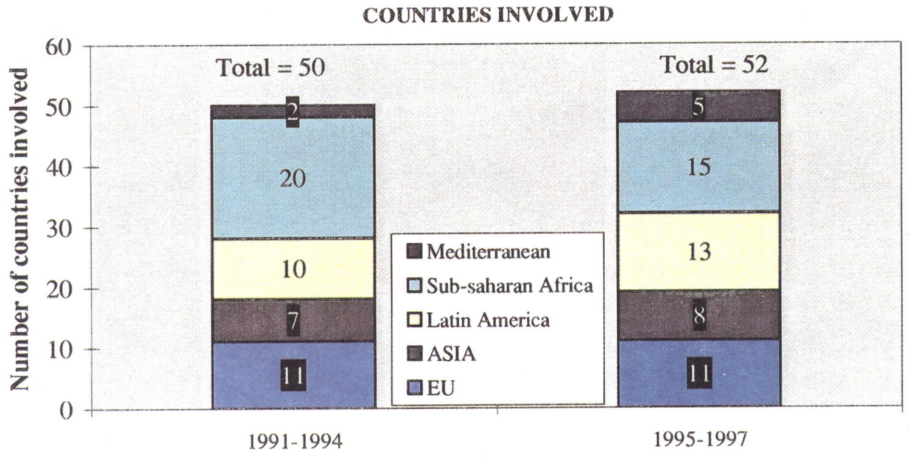
Trends

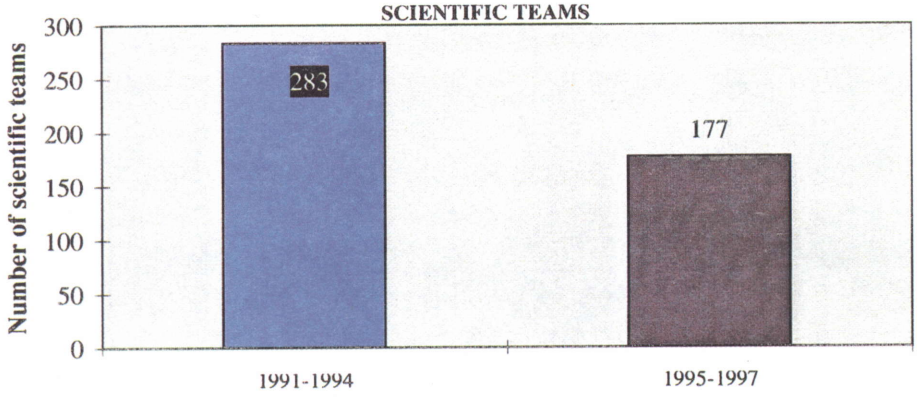
From STD-3 to INCO-DC, more high quality proposals have been selected. New equitable partnerships have been initiated and ongoing collaboration has been reinforced. The increased awareness of the worsening malaria situation has led to the demand for increased efforts to deal with the problem. The challenge has been taken up by the scientific community and the INCO-DC Programme has responded to this by allocating increased levels of support to malaria projects, at the same time maintaining its investment in other areas of health research.

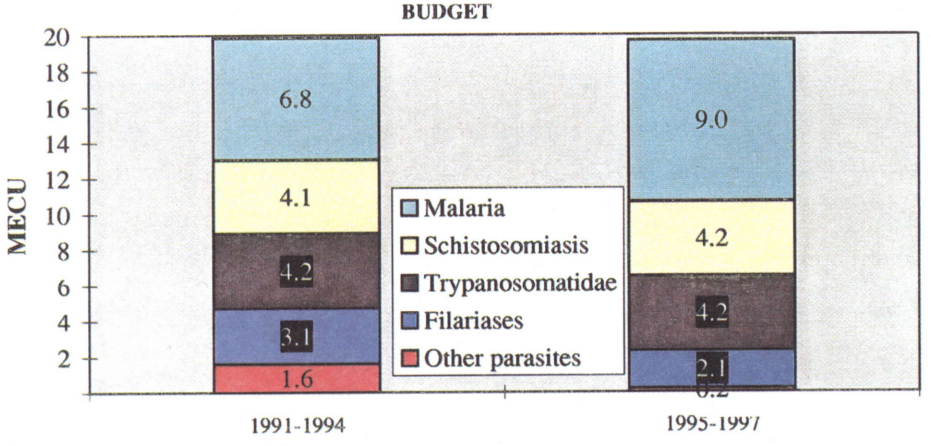
PARASITOLOGY RESEARCH

COUNTRIES, TEAMS, BUDGET

STD 3 **INCO-DC (1st & 2nd call)**







Presentation of EC supported joint research projects (1991-1996)

STD3/INCO-DC: 1st and 2nd Call

Presentation of EC supported joint research projects (1991-1996)
STD3
INCO-DC: 1st and 2nd Call

Areas of interest:

1. Malaria

Contract number TS3*CT910032

BEHAVIORAL STUDIES OF *ANOPHELES GAMBIAE* AND *CULEX QUINQUEFASCIATUS* FOR THE DEVELOPMENT OF ODOUR-BAITED TRAPS, 1

Period: April 1, 1992 - March 31, 1995

Co-ordinator: WAGENINGEN AGRICULTURAL UNIVERSITY,
DEPARTMENT OF ENTOMOLOGY,
Wageningen, The Netherlands (W. TAKKEN)

Objectives

- To study the principal cues that mosquitoes use to identify a human host.
- To analyze how these cues guide mosquitoes to the host.
- To determine the effects of insecticides on host seeking behaviour.

Activities

The target insect will be *An. gambiae* but work on other malaria mosquitoes and on *Cx. quinquefasciatus* will be included.

Host seeking in experimental huts

The rate of entry and exit and movements of mosquitoes into the huts is monitored by video, electric nets and/or infra-red sensors. The temperature, humidity and air currants are recorded at various points within the hut on a data logger. These data are then correlated with the movement patterns of the mosquitoes to elucidate the mechanism of host location. For these experiments two pairs of field assistants are chosen so that in each case one man is relatively attractive and the other unattractive, as determined by previous landing catches. Only the legs of the men are exposed, so that the approach, landing and biting behaviour of the mosquitoes can be recorded on video. The men will otherwise be protected by bed nets and offered anti-malaria prophylaxis under medical supervision.

Landing catch

To determine how accurately landing-catchers reflect the true biting behaviour and host preference of undisturbed mosquitoes, the men will conduct a routine landing-catch in the experimental hut, instead of allowing the mosquitoes to feed freely.

Contract number TS3*CT910032

Attractiveness of human odours

The two field assistants will sleep outdoors in tents and their odours will be pumped into the experimental huts with fans and plastic lay-flat tubing. The odour will be released from the end of the tubing where the assistants would normally lie, at an airflow rate similar to that created by convection currents. An electric net will be placed over the opening of the tube to collect mosquitoes as they arrive at the odour source.

Identification of attractive odours

Skin emanations and expired air will be trapped on solid adsorbents. Successive fractionation should make it possible to start identifying components in the active fractions by standard spectroscopic techniques. In parallel with this, the possibilities for electrophysiological recording from antennae of *An. gambiae* will be investigated, using both EAG and single cell technique. The behavioral effect (activation, orientation and landing) of the most promising candidate odours and combinations of odours will be analyzed first in the laboratory windtunnel and then in the field.

Several types of traps, baited with candidate host odours will be tested in the experimental huts. Paired studies using human-landing catches, light-trap catches and odour-baited traps will be made, in order to assess the relative attractiveness of odour baits compared to the other sampling methods.

Insecticides currently in use for the treatment of bed-nets or as residual on indoor resting sites of mosquitoes, will be tested for their effect on house entry and host-seeking behaviour of *An. gambiae* and *Cx. quinquefasciatus.* As soon as the behaviour in the laboratory is known, this work will be repeated in experiments with huts in the field.

Results

Host seeking in experimental huts

Field studies with the odour-baited entry trap (OBET) continued in 1994 and 1995, both in Burkina Faso and in Tanzania. Also, experiments were done in tent-traps. In both study areas it was found that *An. gambiae s.s.* expresses differences in attractiveness for human hosts, based on odours only. These differences are consistent in time and are expressed both at the individual level and within a group. A study to reveal the nature of these differences suggested that these are primarily due to the concentration of CO_2 in the exhaled breath.

A dose response relationship for the attraction to CO_2 was found for several mosquito species, being clearest for *Mansonia uniformis* and *An. gambiae s.l.*, through in the latter species this relationship did not hold beyond the natural emission rate of CO_2. Furthermore, the Burkina Faso studies showed that about 30% of the attraction of *An. gambiae* to humans was caused by CO_2 and the remaining attraction was caused by body odours. A similar study in Tanzania found that only 8% of the attraction to humans was caused by CO_2 which was not increased by higher levels of CO_2 output. Both studies showed that breath odours (other than CO_2) did not contain important attractants for *An. gambiae s.l.* and that human body odours must account for the high degree of anthropophily in this species. A newly developed odour-baited electric-grid trap, designed to intercept mosquitoes flying outdoors, proved successful in collecting several mosquito species, including *An. gambiae* and *Culex quinquefasciatus.*

Identification of attractive odours

Previously we reported that *An. gambiae s.s.* was attracted to the odours of human feet, and that these could be (partially) removed by washing with bactericidal soap. Further studies on the identification of attractive odours demonstrated that *An. gambiae s.s.* is attracted to the emanations of Limburger cheese. These resemble human foot odour, and caused a strong attraction both derived from natural cheese as well as after extractions in acid. Chemical analysis showed that the active fractions consisted mostly of low-chain fatty acids. The acid fraction and a synthetic mixture of fatty acids, based on the composition of natural fatty acids in cheese, based on the composition of natural fatty acids in cheese, were attractive at very low concentrations. The original extract was repellent. Electro-antennogramme recordings with fatty acids showed a high activity for individual components which was dose-related. Chemical analysis of human sweat, obtained from volunteers in Burkina Faso and Tanzania, showed that human sweat contains similar fatty acids as found in cheese emanations. A preliminary field study with the fatty acid mixture in Tanzania did not confirm the laboratory results, but this may have been caused by inadequate release methodology of the fatty acids.

Effect of insecticides on host seeking behaviour

These studies have not been undertaken due to lack of time and facilities, given the priorities set within the total study.

Contract number TS3*CT910032

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Contract number TS3*CT920101

BEHAVIORAL STUDIES OF *ANOPHELES GAMBIAE* AND *CULEX QUINQUEFASCIATUS* FOR THE DEVELOPMENT OF ODOR-BAITED TRAPS, 2

Period: October 1, 1992 - June 30, 1996

Co-ordinator: UNIVERSITÀ DI ROMA "LA SAPIENZA", INST. DI PARASSITOLOGIA, Roma, Italy (M. COLUZZI)

Objectives

- To study the principal cues that mosquitoes use to identify a human host.
- To analyze how these cues guide mosquitoes to the host.
- To determine the effects of insecticides on host seeking behavior.

Activities

This project is complementary to the project under contract number ST3CT910032 and the two projects are fully integrated.

The testing of host-seeking behavior will be done both on single mosquitoes as well as on groups of mosquitoes subjected to alternative stimuli in the windtunnel. This should also include the study of the effect of mechanical barriers in the approach to the stimuli in an attempt to determine behavioral differences related to endophily. Marking with fluorescent powder will be utilized when comparing different strains or carriers of different karyotypes by mass released in the windtunnel.

It is expected that strain differences are experimentally established and they will be crossed and backcrossed for the genetic analysis of the specific responses and these analyses can eventually proceed up to gene mapping, depending on the results. The link with ISTPAR on genome mapping of *Anopheles gambiae* is expected to contribute to this aspect of the work.

Depending on the outcome of these experiments these strains will also be used in genetic analysis of the selected behavioral differences. The candidate material is from *Anopheles arabiensis* populations showing obvious differences in the field for both the degree of anthropophily and endophily. Other candidate strains already available are various alternative chromosomal variants obtained from polymorphic populations of *Anopheles gambiae* such as the alternative homokaryotypes 2L+ and 2La-.

Contract number TS3*CT920101

Results

Field studies were conducted in Burkina Faso. In a two-choice experiment, using odor-baited entry traps (OBETs), placed side by side outside a rural village, where populations of *An. gambiae s.l.* are abundant during the rainy season, traps baited with human odor or odors from a calf, have shown consistent differences in catch composition. For the human trap the ratios were 52% *An. arabiensis* to 48% *An. gambiae* s.s., for the calf-trap the ratios were 92% *An. arabiensis* to 8% *An. gambiae s.s.*. These results confirm the very high degree of anthropophily of both *An. arabiensis* and *An. gambiae s.s.* in West Africa, and show that odors other than CO_2 contribute to the determination of that preference. They also confirm our previous field observations on odor-mediated differences in behaviour between these two species. In a related field study in Tanzania it was found that during the rainy season *An. arabiensis* was also highly attracted to human odour, to the same degree as *An. gambiae s.s.*. In the latter study it was also found, as in Burkina Faso, that other odours than CO_2 contribute to the attraction to human hosts. However, in Tanzania the role of CO_2 was of less importance than in Burkina Faso. The differences found underline the heterogenetic variance in host-seeking behaviour within members of the *An. gambiae* complex, which may be related to host availability and geographical separation.

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Contract number TS3*CT920044

IDENTIFICATION OF A PROMOTER SPECIFICALLY TRANSCRIBED IN THE GUT CELLS OF ANOPHELES MOSQUITOES FOR THE EXPRESSION OF ANTIPARASITIC AGENTS, I

Period: October 1, 1992 - September 30, 1995

Co-ordinator: UNIVERSITÀ DI ROMA "LA SAPIENZA",
INST. DI PARASSITOLOGIA
Roma, Italy (A. CRISANTI)

Objectives

We propose to perform a series of experiments aimed at the identification of mosquito promoter/enhancer sequences specific for the cells of the intestinal lineage of Anopheles. The candidate genes for conferring a non-vectorial phenotype (anti-gamete/ookinete specific immunoglobulin), against malaria (both human and mouse) will be targeted for expression in mosquito intestinal cells by means of a DNA vector containing the specific promoter sequence. The experimental objectives include:

- identification of mosquito genes that are specifically transcribed in the gut cells;
- identification of the upstream regulatory DNA sequences that drive gut specific transcription;
- transfection of mosquito cell lines with DNA constructs containing a reporter gene under the control of the selected promoter sequences;
- development of a suitable *in vitro* and a laboratory scale assay to determine the tissue specificity of the selected promoter;
- cloning of the coding sequences for the binding region of Plasmodium gamete/ookinete antibodies. The gut specific promoter will be used to develop transgenic and transplanted mosquitoes secreting in the midgut transmission blocking antibodies. The transmission blocking activity of the antibody produced in the mosquito midgut will be assayed in the human *P. falciparum* and in the mouse *P. berghei* models.

Activities

Identification of a promoter sequence specifically transcribed in Anopheles gambiae: gut cells

The genes specifically expressed in the gut cell of the *Anopheles* will be isolated from a λgt 11 cDNA expression library with the help of an antiserum raised against the secretory protein of the gut. The 5' non transcribed promoter region will be isolated from a genomic library using specific cDNA clones. Also the coding sequences of the Aedes trypsin gene will be used to screen the Anopheles genomic library to search for the corresponding promoter. The transcription start site will be identified by primer extension and S1- mapping and the promoter region will be subcloned. The promoter will be functionally defined by deletion mapping and *in vivo* assay. In addition the putative regulatory transcription sequences will be tested for interaction, in gel shift electrophoresis, with protein from *An. gambiae* nuclear extract.

Cloning of the heavy (H) and light (L) chains of gametocyte monoclonal antibody with blocking activity

Mouse hybridomas screening antibodies that both react with gamete/ookinete antigens and have transmission blocking activity will be collected from several laboratories. It is our intention to use antibodies against both *P. falciparum* and *P. berghei* antigens. The variable domains of the heavy and light chains will be amplified from cDNA generated from the secreting cells.

Contract number TS3*CT920044

The coding sequences of the variable regions H and L will be cloned in a eucaryotic expression vector within the invariant regions of the γ and κ chain respectively and expressed in NSO cells, (Orlandi et al 1989). As control the transmission blocking function of the recombination antibody will be tested in the supernatant of transformed NSO cells.

In vivo activity of the putative An. gambiae gut specific promoter

The upstream sequences of the gut specific transcribed gene will be tested on mosquito-derived cell lines for the ability to induce specific transcription and expression. Both a reporter gene (luciferase) and the cloned gamete/ookinete antibody sequence will be cloned in a vector, containing long genomic cDNA sequences of *An. gambiae*, downstream of the putative promoter.

Expected outcome

If the product of the transgene is able to interfere with virus and parasite replication, the resulting mosquitoes should display a non-permissive phenotype for disease transmission.

The identification of a gut promoter would be particularly relevant for the generation of transgenic mosquitoes to be used in the genetic control of the wild type trains. In fact, to be successful the product of the transgene should not impair the environmental fitness, the fertility and the behavior of the mosquitoes. This would be better achieved if the expression of the transgene is restricted, by a specific promoter, to the organ (gut) were most parasites and viruses undergo replication.

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Contract number TS3*CT920139

IDENTIFICATION OF A PROMOTER, SPECIFICALLY TRANSCRIBED IN THE GUT CELLS OF ANOPHELES MOSQUITOES FOR THE EXPRESSION OF ANTIPARASITIC AGENTS, II

Period: January 1, 1993 - December 31, 1995

Co-ordinator: UNIVERSITÀ DI ROMA "LA SAPIENZA",
INST. DI PARASSITOLOGIA, Roma, Italy (A. CRISANTI)

Objectives

- To determine the exact place and time of expression of genes that are expressed in the gut of the mosquito *Anopheles gambiae*, using the heterologous *in vivo* system of *Drosophila melanogaster.*
- To map on the genome of *Anopheles gambiae* a set of molecular landmarks consisting of anonymous cDNAs to sequence expressed in the gut of this mosquitoes.

Activities

This project is complementary to the project under contract number TS3*CT910044 and the two projects are fully integrated.

The study of *Drosophila* genetics during the last decade has led to development of several techniques that are of the utmost importance in the study of this organism. These techniques include both the stable germline transformation (using P-element and *hobo* based vectors) as well as analysis of the transit expression in somatic tissues, that is investigated after the injection of specific constructs into early embryos. In addition, several more experimental procedures are now available, that allow the study the expression of specific genes throughout the different developmental stages of the fruit fly. Thus, *in situ* hybridization and immunochemical stains make the visualisation of gene products (mRNA and protein) feasible in both whole mounts and sections through the animals.

As the above mentioned methods are not yet available for mosquitoes (in particular germline transformation) the temporal and spatial pattern of expression of sequences that are presumed to be active in the digestive tract of *An. gambiae* will be studied using the available techniques in *Drosophila*. Once these genes have been isolated and their putative "control" regions have been identified through "classical" molecular analysis, their pattern of expression will be investigated in *Drosophila*. The idea is to dissect the *cis*-control tract, so that potentially useful elements can be identified for the future construction of chimeric plasmids to be expressed, in the midgut of the mosquito.

The rationale for the cross-species experiments that are proposed is the fact that it has been previously shown that control regions of specific genes can be recognised by the regulatory proteins of animals that are separated even by several hundred millions of years of evolution. Here 5'-non-transcribed sequences of the mosquito genes will be fused to appropriate marker genes (e.g. the *lacZ* gene from *E. coli*) and the expression pattern will be established in *Drosophila* after germ line transformation, using P-element based vectors. Similarly, the same or analogous constructs will be used for transient expression experiments. By using chimeric plasmids encompassing different upstream segments of genes to be analyzed the necessary and sufficient *cis*-controlling sequences will be determined. For this, in both of these lines of investigation, serial sections as well as whole mounts of Drosophila will be examined throughout the course of development of the animal.

Identification of Anopheles genes expressed in the gut upon blood feeding

Serine proteases are among the enzymes which play a crucial role during the digestion of the blood meal in the gut of mosquitoes. Experimental evidence indicates that trypsin activity can be recovered in the gut of Anopheles mosquitoes a few hours after blood feeding. We thought that the analysis of the genomic organization of the trypsin genes may lead to the isolation of a gut-specific, inducible promoter for the expression of anti-parasitic agents in transgenic mosquitoes.

According to the objectives of the proposal, we have performed a series of experiments aimed at identifying *Anopheles gambiae* trypsin genes and at studying their genomic organization.

cDNA cloning and sequence analysis

The PCR product obtained with the primers deduced from conserved trypsin sequences was used as probe to search for full length cDNA clones in a λgt11 expression library developed using cDNA generated from mRNA of blood fed *A. gambiae*. The inserts of a number of positive λgt11 plaques were cloned in the EcoRI site of the pDS56/RBSII, 6xHis/E- polynker. Two types of clones were identified according to the restriction pattern of the two previously cloned PCR fragments. Sequence analysis of full length cDNA clones of each type, Antryp1 and Antryp2, showed that they encompassed the respective partial sequences of the cloned PCR products. Antryp1 and Antryp2 revealed open reading frames (G/C-content of 60% and 59% for Antryp1 and Antryp2 respectively), coding for a polypeptide of 274 and 277 amino acids respectively. The analysis of the translated product of Antryp1 and Antryp2 indicated that both clones encode for two distinct polypeptide showing 75% homology at the amino acid level. Moreover, both polypeptides are highly homologous to known trypsin sequences.

Contract number TS3*CT920139

Genomic organization of the Anopheles gambiae trypsin genes

To determine the genomic sequences of Antryp1 and Antryp2, a genomic *A. gambiae* λEMBL3A library was screened with the same probe that was used for the isolation of the λgt11 clones. Several overlapping λEMBL3A clones were obtained, two of them, Ty 3.3 and Ty 4.1 containing both trypsin sequences. Hybridisation experiments carried out on the genomic clones and on *A. gambiae* DNA digested with a set of restriction enzymes revealed several bands that could not be explained by the presence of Antryp1 and Antryp2 only. We have sub cloned and sequenced the internal BamHI fragments of the genomic subclone, containing Antryp1 and Antryp2, confirmed the respective cDNA sequences and indicated that the coding sequences of both genes were not interrupted by any intron. Moreover, five additional trypsin related genes were found in proximity of Antryp1 and Antryp2, also these genes did not contain any intron. Contiguity and relative orientation was confirmed by further restriction fine mapping of the genomic clones TY3.3 and TY4.1 which was in accordance with the results obtained by Southern analysis of genomic clones. In addition, contiguity was proven by PCR performed on TY3.3 using specific primer pairs, followed by digestion of the PCR products with restriction enzymes specific for each fragment.

Blood meal induced expression of the trypsin genes Antryp1 and Antryp2

The expression of the trypsin genes in the mosquito was assessed by Northern analysis using total RNA extracted from female mosquitoes at different time points after blood feeding. As control, RNA from male mosquitoes, fourth instar larvae, pupae and non-fed female mosquitoes were analyzed. As probes the oligonucleotides pTy1-S and PTy2-S that have unique sequences specific for Antryp1 and Antryp2 respectively were used. To reduce the possibility of cross hybridisation of the probes on similar sequences, Ty1-S and pTy2-S were derived from one of the most polymorphic regions of the trypsin genes. In Nothern blot, both probes hybridized with a RNA species migrating around 1kb with an estimated transcript length of about 950 bases. Adult females and to some degree pupae, showed a small amount of Antryp1 transcript. After blood feeding, in female mosquitoes transcription of Antryp1 steadily increased and reached its peak after 24h. The amount of Antryp1 mRNA was decreased 40h after the blood meal. Antryp2 transcripts could be shown only in female mosquitoes between 8 h and 24 h after blood feeding. The amount of transcript induced is at the detection limit of end labelled oligomers in Northern analysis. Induction of the Antryp2 gene could be demonstrated much clearer in amplification experiments using as template cDNA generated from blood fed mosquitoes. Under this experimental condition it was possible to demonstrate that Antryp2 is expressed only in female mosquitoes and that its induction is tightly controlled.

Contract number TS3*CT920139

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Contract number TS3*CT930236

NETWORK FOR FIELD RESEARCH ON AFROTROPICAL MALARIA VECTORS

Period: October 1, 1993 - September 30, 1996

Co-ordinator: UNIVERSITÀ DI ROMA "LA SAPIENZA",
INST. DI PARASSITOLOGIA
Roma, Italy (M. COLUZZI)

Objectives

General objectives

- To improve the knowledge of malaria vectors and transmission in subsaharan Africa by introducing new methods (genetic, molecular, immunological) for the study of the vector and the parasite in the vector.
- To predict and monitor variations in the malaria vector system related to ecological changes induced by urbanization, desalinization, deforestation and irrigation of arid areas.
- To obtain and assist African scientists in improving research capabilities applied to malaria entomology and vector control.

Specific objectives

- To evaluate the influence of salt vs. fresh water environments in the frequency of *An. gambiae* and *An. melas* and their chromosomal variants in West African coast localities.
- To determine the redistribution, bionomics, population and vector status of the newly described Mopti chromosomal form of *An. gambiae* and of its inversion karyotypes.
- To determine the population structure and bionomics of *An. funestus* with particular reference to intrapopulation genetic heterogeneities.
- To evaluate the role of *An. pharoensis* and *An. rufipes* in the transmission of *P. falciparum* and to analyze sibling species within these taxa.
- To implement large-scale use of molecular probes for the identification of sibling species on the *An. gambiae* complex in malaria epidemiology studies.

Activities

The present project implemented research and training activities already in progress within STD1 and STD2 programs coordinated by ISTPAR-Rome. These activities were focused on specific research objectives emerged from previous results while the training was expanded to Portuguese-speaking countries via the participation in the network of the IHMT of Lisbon. The DC partners co-participants in the proposal were laboratories in Mali (Département d'Epidémiologie des Affections Parasitaires, "DEAP"), in Madagascar (Etablissement d'Enseignement Supérieur des Entomologiques, "OCCGE"), where good research facilities are available together with experienced African leadership trained under STD1 and STD2. However, other African countries were involved, namely:

* Burkina Faso, where the "Centre National de Lutte contre le Paludisme" (CNLP) financed by Italian Cooperation was a focal point for research and training.
* Guinea-Bissau, Sao-Tome and Cabo Verde, where IHMT implemented research and training.

Moreover, the network collaborated with various groups working in field research on Afrotropical malaria vectors, particularly with ORSTOM-Institut Pasteur in Senegal.

Results

All the above specific objectives were pursued and further progress towards the achievement of the general objectives were obtained as follows:

Research on influence of salt vs. fresh water environments was carried out in Guinea-Bissau by IHMT and ISTPAR.

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Different seasonal and spatial distributions of *An. melas* and of *An. gambiae* Bissau and Savanna chromosomal forms were recorded and their relative contribution to *P. falciparum* inoculation rates was evaluated. The importance of 2Rn inversion polymorphism in *An. melas* in relation to the distance from the sea has been confirmed. This chromosomal variation appears adaptive for tide-dependent larval breeding places.

Ecological genetic studies were carried out by DEAP and ISTPAR on the chromosomal form Mopti of *An. gambiae* in Mali in samples from a 16-locality transect. The 2R polymorphism showed wide variation and highly significant correlation with both temporal and spatial climatic differences. Mosquitoes homokaryotypic for 2Rbc were found to be the actual dry season and arid area breeders. Only the chromosomal form Mopti fully exploits the breeding opportunities during the dry season and is able to displace by competitive exclusion the other taxa of the *An. gambiae* complex, namely *An. arabiensis* and *An. gambiae* Savanna.

Investigations in Burkina Faso carried out by ISTPAR and CNLP have brought the discovery of sibling species within the taxon *An. funestus*, the most important malaria vector in Africa South of the Sahara after *An. gambiae*. The standard arrangement of the polytene complement prevails in one of the taxa while the other was found to be highly polymorphic. The latter, characterized by a higher vectorial capacity, was the only one present in Madagascar where it hibernates at the larval stage on the Plateaux (Antananarivo area). Both taxa were identified in Senegal and in Mali.

Various samples of *An. pharoensis* were examined and CSP positive specimens were detected in Burkina Faso. The evaluation of the vector role of this species requires further investigations.

The large scale use of polytene chromosome analysis and of available diagnostic molecular probes for the study of malaria vectors was successfully implemented and specific training was provided by ISTPAR for a total of 16 months/man. Three trainees were from IHMT, two from DEAP, one from EESS and one from OCCGE.

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Contract number IC18*CT960030

STUDIES ON MOSQUITO VECTORS IN WEST AFRICA, AIMED AT MALARIA EPIDEMIOLOGY AND CONTROL

Period: December 1, 1996 - November 30, 1999

Co-ordinator: CENTRO DE MALARIA E OUTRAS DOENCAS TROPICAIS (IHMT), Lisbon, Portugal (V.E. DO ROSARIO)

Objectives

- To determine the distribution, bionomics, population structure and vectorial capacity of malaria vectores in S.Tomé and Equatorial Guinea.
- To implement the large scale use of molecular probes for the identification of anopheline sibling species.
- To identify human parasite species in mosquitoes by PCR using different methods of DNA extraction.
- To perform longitudinal studies on the population dynamics of mosquito vectors in association to interventions.

Activities

* Collections will be carried out by teams already trained, in both islands, and under supervised visits. These will take place, twice a year for the initial collection of data, followed by regular samplings throughout the year, aimed at studies of distribution and bionomics.
  Locations of collections will be made preferably, in relation to a) intervention sites e.g. drainage and local use of mosquito bed nets vs controls; b) randomly determined locations. These are being discussed at this stage.
  Population structure will be assessed by specific inversion kayotype studies as well as PCR classification.
  During the first year, training on these and other required techniques will take place at Lisbon and Rome. Implementation in the field of these techniques and establishment of functional laboratories in the field will take place in the first year.
* Validation of field sampling methods, including CDC miniature light traps, indoor resting collections, exit window traps and human landing collections, for estimating entomological inoculation rates (EIR), for the different vector species, will be undertaken and the most reliable and cost effective method will be adopted for long term studies.
  Vectorial capacity will be further evaluated by standardized ELISA assays for NANP Ag.
  Whenever required, age grading studies will take place, after analysis of the first years results.
* PCR methodology will be applied mainly for comparative studies from regions within each island and these will aim specifically to the *Anopheles gambiae* complex of vectors.
  PCR studies aimed at identifying human malaria parasites will take place in order to evaluate transmission of the 4 existing malaria parasites in both islands. These are carried out from both oocysts and salivary glands, from naturally fed mosquitoes, collected from a) patients with identified gametocytes by optical microscopy, b) from a random sample of the population.
  PCR studies for the identification of human malaria parasites require preliminary laboratory assays in order to improve DNA extraction from parasites and thereby reduce technical difficulties due to excessive mosquito material. These studies will be initiated in the laboratory in Lisbon.
* Interventions are planned, locally, aimed at malaria control. These include the utilization of impregnated bed nets (Sao Tomé and Equatorial Guinea), and drainage of some areas in Sao Tomé where malaria is highly prevalent. Both are being implemented now and studies on the dynamics of vector populations will be carried out according to methodologies described above.

Contract number IC18*CT960030

Expected outcome

⇒ Training of personnel from SaoTomé e Principe and Equatorial Guinea, in Lisbon (PCR) and Rome (Cytogenetics).
⇒ Workshop in Equatorial Guinea, on the project aims and methodologies will take place in March/April 1997.
⇒ Local laboratories, fully equiped, able to carry out studies on vector identification by cytogenetics and DNA extractions.
⇒ Determination of the best mosquito collection methods to apply in the field will be determined and applied within a standardized work programme. Longitudinal time series analysis of (daily) collections from individual houses will be completed after 2-3 years.
⇒ Identification of malaria vectors and parasites by PCR and cytogenetics, on both islands, after 18 months.
⇒ Mapping of collection sites and vector distribution by GPS (Global Positioning System) established within the first year.
⇒ Integration of epidemiological morbidity data with entomological information by the second year.
⇒ Integration with parallel studies on drug resistance in Sao Tomé and Equatorial Guinea.
⇒ The relative risk of malaria transmission, by time of night, area, season and vector species will be determined.
⇒ Appropriate vector control strategies, based on the assessment of these risks, will be defined and implemented in defined sites.

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Contract number IC18*CT970211

IDENTIFICATION AND CHARACTERISATION OF MALARIA VECTORS IN SOUTH EAST ASIA: A PREREQUISITE FOR APPROPRIATE VECTOR CONTROL

Period: December 1, 1997 - November 30, 2000

Co-ordinator: INSTITUTE OF TROPICAL MEDICINE, Dept. of Parasitology, Antwerp, Belgium (M. COOSEMANS)

Objectives

General objectives

- To develop in Southeast Asia appropriate vector control measures based on the correct identification of the vector species involved in malaria transmission and on their behaviour in order to reduce malaria incidence in populations at high risk and to prevent malaria epidemics by early detection and control.

Measurable specific objectives

- To elucidate the epidemiological relevance of heterogeneities in vector populations and the sibling species of malaria vectors in different physico-geographical regions of Southeast Asia.
- To develop improved methods for vector species identification and to obtain clear criteria that can be used to define malaria risk areas; this will help in the choice and implementation of preventive measures.
- To study the practical implications of the genetically defined sibling species in relation to vector behaviour, vectorial capacity.
- Capacity building by exchange of technologies and collaborative training in research methodology.

Activities

The study will be performed in three physico-geographical regions of Vietnam (northern, central and southern), in Laos and in Cambodia.

Baseline ecological and malaria epidemiological data will be collected for each study site.

Mosquitoes (adults and larvae) will be collected in each study site applying multiple collection methods during the first year of the project.

Different experimental procedures to analyse the field samples will be applied: morphology, isozyme electrophoresis, RAPD-PCR, PCR-RFLP and ELISA.

Isozyme electrophoresis will be applied to morphologically identified forms (morphotypes) in order to confirm species identification, and to discriminate individual members of species complexes. This technique will provide information on the extent of genetic variation between and within different populations. This will allow us to detect intrinsic barriers to gene flow by the demonstration of a lack of heterozygotes. The enzymatic markers will be tested for their ability to distinguish potential cryptic species and subsequently used to investigate species relationships relevant to vector biology and transmission.

Molecular techniques will be applied for the development of simple and reliable tools for species identification; e.g. screening of RAPD-PCR primers; amplification of variable rDNA regions and RFLP analysis. The molecular markers will be tested for their ability to distinguish cryptic species identified by isozyme electrophoresis.

Contract number IC18*CT970211

Expected outcome

The project will assess the species status of the three major malaria vectors in Southeast Asia and will evaluate the role of the individual species in malaria transmission. This will lead to considerable improvement of the vector control activities by focusing the control measures on the efficient vectors with the appropriate tools and in relation to their behaviour. Moreover, a better evaluation of the intervention, using the new identification methods, is an additional benefit. It would also be possible to estimate the risk of environmental changes on vector distribution.

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Contract number IC18*CT970244

DEVELOPMENT AND FIELD APPLICATIONS OF MOLECULAR PROBES FOR THE STUDY OF AFROTROPICAL MALARIA VECTORS

Period: December 1, 1997 - May 31, 2001

Co-ordinator: FOUNDATION FOR RESEARCH & TECHNOLOGY HELLAS,
Inst. of Molecular Biology & Biotechnology,
Heraklion, Greece (C. LOUIS)

Objectives

- The development of rDNA-based molecular markers for each of the taxonomic units shown within *An. gambiae* s.s. and *An. funestus* s.s.
- The evaluation of the new diagnostic tools on field-collected material typed by polytene chromosome analysis.
- The physical isolation and cloning of the DNA surrounding the breakpoints of inversion *2La*.
- The sequence determination of specific DNA segments linked to *2La* and their use in the karyotype identification of field-collected malaria vectors and in phylogenetic studies.

Activities

This proposal focuses on the molecular analysis of the *Anopheles gambiae* and the *An. funestus* complexes, the malaria vector system in Sub-Saharan Africa. The sibling species in the complexes are defined by the presence or absence of specific inversions detected on polytene chromosomes. Furthermore, there are indications that additional taxa carrying typical sets of polymorphic chromosomal inversions and characterized by variable degrees of intergradation, are members of these complexes. The long term goal is to undertake an in depth study of the genetic structure and dynamics of field populations of *An. gambiae* and *An. funestus* and to attempt to correlate specific recognizable genetic polymorphisms with vector biology, in order to provide a better description of this powerful vector system and to obtain reliable data towards its monitoring and control.

We propose to undertake a molecular genetic analysis of the inversion polymorphisms using molecular probes. The primary objective of the present study is the development of specific PCR-based diagnostics based on both rDNA (for both *An. gambiae* and *An. funestus*) and cloned inversion breakpoints for the *2La* inversion of *An. gambiae*. As a parallel objective, we wish to analyze the phylogenetic relationship of chromosomal forms of *An. gambiae* and to obtain information on the monophyletic origin of the inversions and on introgression and gene flow among different inversions.

Expected outcome

⇒ The development of rDNA-based molecular markers for each of the taxonomic units shown within *An. gambiae* s.s. and *An. funestus* s.s.
⇒ The evaluation of the new diagnostic tools on field-collected material typed by polytene chromosome analysis.
⇒ The physical isolation and cloning of the DNA surrounding the breakpoints of inversion *2La*.
⇒ The sequence determination of specific DNA segments linked to *2La* and their use in the karyotype identification of field-collected malaria vectors and in phylogenetic studies.

Contract number IC18*CT970244

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Contract number TS3*CT920103

PLASMODIUM GENOME - STRUCTURE AND DYNAMICS

Period: October 1, 1992 - March 31, 1995

Co-ordinator: UNIVERSITY OF EDINBURGH, INST. OF CELL, ANIMAL AND POPULATION BIOLOGY, Edinburgh, United Kingdom (D. WALLIKER)

Objectives

The overall objective of the project has been to understand the mechanisms which give rise to the genetic diversity seen in populations of malaria parasites. The project has involved field studies on the most important species affecting humans, *Plasmodium falciparum*, to determine the extent of polymorphism of certain genes among the parasites of small communities and to examine the role of cross-mating between parasites in generating this diversity. In the laboratory, studies on the organization of the genome have been carried out with both *P. falciparum* and the rodent malaria model *P. berghei,* with particular emphasis on the organization of repetitive DNA elements and their role in generating chromosomal re-arrangements.

Activities

Genetic polymorphism in natural P. falciparum populations

Much work in recent years has established that there is considerable genetic diversity in populations of *P. falciparum*, even among the parasites of small communities. Certain genes, notably those encoding antigens, exist in such populations as a complexity of allelic variants, and many patients contain mixed infections of more than one parasite clone. The significance of these findings for control measures, especially those based on vaccination, has yet to be established.

The work undertaken during this project has been to examine the diversity of three *P. falciparum* genes among parasites in villages in Papua New Guinea (PNG). The objectives were (i) to determine whether there were differences between the frequencies of allelic variants of these genes among the parasite populations of different villages in a single region (ii) to investigate what changes occur in these antigens during the course of an infection in individual patients, and (iii) to determine the frequency of crossing between different parasite clones during mosquito transmission.

Diversity of antigen genes in blood infections

Methods based on the polymerase chain reaction (PCR) have been devised to identify alleles of merozoite surface proteins MSP-1 and MSP-2. we have concentrated most effort on MSP-2 because this antigen is expected to form part of a malaria subunit vaccine to be tested in this area in the near future.

Results

A survey of the MSP-2 alleles of 304 people living in the Wosera region of PNG revealed 38 alleles which could be discriminated by this technique, 33 of which have proved to be novel alleles. Twelve alleles have also been sequenced, six of which have been found to belong to the so-called "FC27" allelic family of this antigen, and six to the "3D7" family. All sequence data are to be analyzed with respect to genetic distance, structure, and periodicities of the repeats, in order to trace how genomic re-arrangements may have occurred during the evolution of this group of alleles.

Information on parasite prevalence as measured by PCR in these individuals was compared with prevalence as measured by microscopy. An important result of this comparison, which could be important for mathematical models of malaria transmission, was that no difference in parasite prevalence was found between children (48%) and adults (47%), whereas by microscopy parasites were detected only in 23% of adults and 32% of children. Furthermore, in more than 50% of all samples positive for *P. vivax* and *P. malariae,* an underlying *P. falciparum* infection was detected by PCR.

The data on MSP-2 genotypes were combined with immunological and epidemiological data collected from the same individuals at the same time. 214 adults were analyzed for antibody and T-cell response against three different MSP-2 antigen preparations. No correlation between antibody or T-cell response and parasite genotype was detected. An attempt was also made to determine whether there was any differential distribution of MSP-2 genotypes in symptomatic and asymptomatic patients infected with *P. falciparum* in case a control study of 227 individuals. Parasites of FC27 genotype were twice as likely to be found in symptomatic as in asymptomatic people.

The overall conclusion of this work was that crossing between parasites was clearly occurring, although at a lower frequency than might be expected from the number of multiclonal infections. This suggested that only a limited number of the clones detected among the blood infections were producing gametocytes infective to mosquitoes. However, it was of interest that no linkage disequilibrium could be detected among the genes studied in the blood infections. Thus the comparatively low degree of crossing in this region was nonetheless sufficient to produce linkage equilibrium in the population of blood parasites.

Karyotype polymorphism

Correlation between chromosome size polymorphisms and the presence or absence of specific functions

Deletions resulting in measurable changes in the sizes of chromosomes of malaria parasites, detectable by pulsed field gradient gel electrophoresis (PFG), are frequently seen in parasites maintained under both *in vitro* and *in vivo* conditions. In some instances, it has been possible to correlate such changes, notably those involving terminal deletions, with the loss of apparently dispensable functions. For example, a deletion polymorphism affecting chromosome 9 of *P. falciparum* has been described, the shorter vision of which involved a terminal deletion which was associated with loss of cytoadherence and poor gametocyte production.

This phenomenon has been investigated in more detail by comparing a number of isogenic cloned lines of *P. falciparum* differing in their gametocyte production. These lines were as follows: (i) seven clones lacking gametocytes which had been derived from the 3D7 gametocyte-producing clone, (ii) clones HB3sel4 (possessing an intact chromosome 9) and clone HB3 (short chromosome 9), and (iii) clone 1776sel8 (intact chromosome 9) and clone C10 (short chromosome 9).

With regard to (i), no obvious size differences were observed between chromosome 9 of any of the seven clones and of the parent 3D7 clone; thus, in this instance, the function impairment (lack of gametocytes) could not be correlated with major deletion or re-arrangement events. With regard to the HB3 series of clones, it appeared that the fraction of parasites bearing a deleted form of chromosome 9 rapidly outgrew the remaining parasites in the cultures. Intact chromosomes are found in the fraction of parasites which develop into gametocytes.

These studies also made use of a gene (Pfg27) expressed early in gametocyte development. Expression of this gene could not be detected in the various gametocyte-less clones studied, in those with deletions in chromosome 9, or in three of the 3D7 derivatives with intact chromosomes. This indicated that these clones were affected very early in their gametocyte development. A structural analysis of the genomic DNA region 5' to the Pfg27 gene revealed that a sequence of 10-15kb adjacent to the Pfg27 promoter is highly polymorphic in the 3D7, HB3sel4 and C10 clones. Some of these polymorphisms might be related to an anomalous production of Pfg27 transcripts in asexual parasites seen in clones C10 and 1776sel8. The region upstream of Pfg27 has also been analyzed in two of the gametocyte-less derivatives of 3D7. A comparison with the parent clones showed that no major structural alteration had occurred in this region during the loss of gametocyte production.

Subtelomeric structures

Evidence is accumulating that genes determining essential functions in Plasmodium are safely placed in the central regions of chromosomes, while genes determining dispensable functions under non-selective conditions are located in the distal regions of chromosomes, which are subject to frequent deletions or re-arrangements.

It is well-known that subtelomeric regions of chromosomes are unstable, but the structural basis of their high degree of polymorphisms is not understood. Species-specific repetitive units are often maintained at the ends of several chromosomes, and are believed to enhance the occurrence of inter-chromosomal recombination events. Results obtained by the Rome group under a previous STD contract showed that in *P. berghei* these subtelomeric repeated sequences are regularly intercalated with telomere-related sequences.

The organization of the subtelomoeric structure present on many *P. falciparum* chromosomal ends thus appears to be substantially different from the one present in *P. berghei*. In particular, there is no evidence of internal telomeric stretches in *P. falciparum*. Homologous recombination would not appear to be particularly favored along the rep20 region, where homology between 21-bp units is limited to not more than 8-9 contiguous base pairs. Exchanges of subtelomeric sequences between non-homologous *P. falciparum* chromosomes might be predicted to occur more frequently in other portions of the sub-telomeric structure, where long regions of homology are present.

However, exchanges in non-repetitious regions would have no detectable effect on chromosome size. From the available evidence, it can be stated with confidence that rep20 is not involved in fundamental functions such as chromosome replication or chromatid segregation, since the whole region can be lost without serious impairment in cell viability.

Diversification of telomeric tracts in P. berghei

Telomeric sequences in Plasmodium result from the irregular alternation of two basic repeats TTTAGGG and TTCAGGG. The length of the telomeric structure, i.e. the number of telomeric heptanucleotide repeats, appears to be constant during mitotic parasite propagation. By sequencing sibling telomeric clones derived from a *P. berghei* chromosomal extremity, it was shown previously that their proximal portions start with identical sequences of the two types of repeats, and that sequence divergence occurs from variable breakpoints. The results suggested that frequent terminal deletions followed by random distributive addition of the two repeat versions is responsible for telomere turnover, in a mechanism of telomere length regulation.

These studies have been extended under the present contract. The breakpoint frequency distribution has been studied in a new set of sibling telomeric clones. The results confirm the suggested hypothesis and show that 90% of the telomeric structure is subject to events causing abrupt changes in the sequence of telomeric repeats.

This indicates that, in the dynamic equilibrium governing steady-stage telomere length, shortening cannot be due to incomplete replication. Rather, terminal deletions appear to be quite frequent events, their frequency rapidly increasing in the outward direction over the entire telomeric tract.

Summary and conclusion

New information has been obtained on the diversity and frequencies of alleles of antigen genes in natural populations of *P. falciparum* in Papua New Guinea. An important result from this field study has been the determination of the frequency of cross-mating between parasite clones in wild-caught mosquitoes; this has implications for predicting how parasites in nature may respond to control measures based on chemotherapy and vaccination. In addition, the laboratory studies on *P. berghei* have provided new information on the organization of sub-telomeric regions of chromosomes. This will undoubtedly lead to some understanding of the genetic mechanisms by which genome re-arrangements occur in malaria parasites. Subjects which remain unfinished include the isolation of candidates for transposable elements, and details of the process of recombination at meiosis.

In the course of this project, all major techniques of molecular genetics have been introduced to the Madang laboratory of the PNG Institute of Medical Research. A laboratory technician from Papua New Guinea has been trained in basic molecular biological techniques, i.e. methods of DNA isolation, Southern blotting and hybridisation, molecular cloning, phage and plasmid mini-preparations, and restriction mapping. Visits of the partners to each other's laboratories have taken place, and solid research collaborations established.

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Contract number IC18*CT960052

REGULATION OF DEVELOPMENT IN MALARIA PARASITES

Period: January 1, 1997 - December 31, 1999

Co-ordinator: UNIVERSITY OF LEIDEN, DEPT. OF PARASITOLOGY, Leiden, The Netherlands (C.J. JANSE)

Objectives

- The investigation of the regulation of parasite development through studies on 1) gene promotor structure, strength and stage specificity and on 2) function of the proteins encoded by the genes under the control of these promoters.
- The genes of study are: *P. falciparum*, Glycophorin binding protein (GBP) 130, Na+/H+ transport protein; from *P. berghei*, PBS21, pbB7, rRNA units (A-D), 150 family, crk2, EF-1a; from *P. vivax*, crk2, EF-1a, rRNA.
- Development of plasmid systems based upon the *Tet* repressor which will allow the inducible expression of cloned genes in different species of *Plasmodium*.
- Where feasible, to develop resources to facilitate the isolation and characterisation of genes encoding proteins with a specific role in development during gametocytogenesis and throughout the mosquito phase of the life cycle in *P. vivax* and *P. berghei*.

Activities

* Where appropriate, to clone and complete the full characterisation of the named genes, their sequence, expression and comparative structures.
* Studying the structure and function of promoters of transcription of RNA polymerases I and II in both *P. berghei* and *P. falciparum* using stable and transient transfection technologies.
* Investigating the species specificity of promoter structure through an investigation of the ability of defined promoter regions of genes isolated from *P. vivax* to accurately control transcription in *P. berghei*.
* Developing plasmid systems based upon the *Tet* repressor which will allow the inducible expression of cloned genes in different species of *Plasmodium*.
* Initiating a study of structure/function relationships of specific parasite structures through gene mutagenesis, replacement, over-expression and/or knock out to establish the role of the protein.
* Studying the control of gene expression in *P. berghei* at the post-transcriptional level in female gametocytes using the Pbs21 (female gametocyte specific), 150 gene family (variant 3' UTR) and pbB7 (nuclear protein gene) as paradigms.

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* Where feasible developing resources to facilitate the isolation and characterisation of genes encoding proteins with a specific role in development during gamecytogenesis and throughout the mosquito phase of the life cycle in *P. vivax* and *P. berghei.*

* To continue to expand the investigation of the relationship between genome organisation and sexual development.

Expected outcome

⇒ The programme should provide a functional analysis of the genes under study and thus provide insights into their role during the complex development cycle of *Plasmodium.*

⇒ An insight will be gained into the functional structure of stage specific promoters of gene transcription. This can be expected to include an identification of those elements which dictate stage- and sex-specificity and those which direct basal transcription.

⇒ The study will provide reagents and materials, which are essential for further studies on the sexual development of *Plasmodium.*

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Contract number IC18*CT960066

ANALYSIS OF *VAR* GENES FROM *P. VIVAX AND P. FALCIPARUM*

Period: January, 1 1997 - June, 30 1999

Co-ordinator: ZENTRUM FUR INFEKTIONSFORSCHUNG, UNIV. WÜRZBURG, Würzburg, Germany (M. LANZER)

Objectives

- to determine the genomic organization of *var* genes in *P. falciparum*
- to examine the mechanism of differential *var* gene expression in *P. falciparum*
- to examine the adhesive phenotypes of specific *var* gene variants
- to clone *var* gene homologous genes from *P. vivax*
- to study the genomic organization of *var* gene homologues in *P. vivax*

Activities

* Production of a *P. vivax* YAC library and the assortment into contigs. A representative YAC library for *P. vivax* will be constructed. The DNA material will be obtained from patients and from primates infected with an Indian isolate of *P. vivax.*

* Assortment of *P. vivax* YAC clones into chromosomal contig maps. Chromosomal YAC contig maps will be generated using a 'top down' approach based on advanced filter hybridization strategies. YAC filters will be screened with random cDNA clones from *P. vivax.*

* Isolation of *P. vivax var* genes. The UNIEBP primers developed to amplify *P. falciparum var* genes will be used to identify and clone homologous genes from *P. vivax.* The production of full-length gene sequences from *P. vivax* will be useful for comparison to the *P. falciparum var* genes, particularly for functional comparison as it is believed that *P. vivax*-infected erythrocytes do not adhere to host endothelium.

* Production of a 3D7 clone tree and cloning of expressed *var* genes. To study the mechanism of *var* gene switching in *P. falciparum*, a clone tree from the *P. falciparum* isolate 3D7 will be generated by limiting dilution. The resulting clones will be analyzed for antigenic similarity using the mixed agglutination reaction with hyperimmune sera. Using RT-PCR, the expressed *var* gene will be identified and sequenced. The sequence will be correlated with the adhesive properties of this variant.

* Mapping a rosetting locus in *P. falciparum.* Using the existing progeny from the genetic cross between the rosetting clone Dd2 and the non-rosetting clone HB3 we will follow the segregation of the rosetting phenotype in this cross and determine a candidate locus. This candidate locus will be screened for the possible expression of *var* genes, to explore the possibility that rossetting is mediated by a particular *var* gene variant.

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* Genomic analysis of *var* genes. Using existing, and yet to be constructed, YAC contig maps of *P. falciparum* and *P. vivax* chromosomes, the genomic location and organization of the *var* gene family will be studied in both *P. vivax* and *P. falciparum*.

* Analysis of *var* genes in field samples (*P. vivax* and *P. falciparum*). To determine the degree of *var* gene variability in field populations, longitudinal studies will be carried out in Brazil and India. The expressed *var* gene variants will be determined by RT/PCR amplification using the UNIEBP oligonucleotides as primers. The resulting fragments will be cloned and sequenced. This will generate a data set of expressed *var* genes within given spatial and temporal locations.

Expected outcome

⇒ The study will provide a better understanding of the mechanisms of differential *var* gene expression.

⇒ The study will correlate defined adhesive properties with particular *var* gene variants

⇒ The study will show the diversity of *var* gene variants in the field.

⇒ The study will provide tools, such as YAC libraries and chromosomal contig maps, that will be useful in other areas of malaria research.

⇒ The study will explore the existence of *var* genes in *P. vivax*.

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Contract number IC18*CT960071

PLASMODIAL CHROMATIN: STRUCTURE AND FUNCTION

Period: January, 1 1997 - June, 30 1999

Co-ordinator: ZENTRUM FUR INFEKTIONSFORSCHUNG, UNIV. WÜRZBURG, Würzburg, Germany (M. LANZER)

Objectives

- to identify *P. falciparum* centromere sequences
- to analyze spatial and temporal chromatin changes and their effect on transcriptional activity in *P. falciparum*
- to develop an *in vitro* plasmodial telomerase assay
- to identify and clone the *P. falciparum* telomerase including its RNA subunit
- to characterize subtelomeric domains in *P. vivax*
- to identify and clone non-histone nuclear proteins
- to identify origins of replication

Activities

* Development of an *in vitro P. falciparum* telomerase assay. An *in vitro* assay for plasmodial telomerase activity will be established. Different oligonucleotides will be tested for function as templates for the plasmodial telomerase. The effect of inhibitors on *in vitro* telomerase activity and *in vivo* parasite viability will be tested.

* Cloning of the plasmodial telomerase. The gene encoding the protein subunit of the plasmodial telomerase will be cloned a) using a structural approach, based on conserved telomerase sequences, b) by screening a *P. falciparum* expression library with antibodies to the Tetrahymena telomerase. The RNA subunit of the plasmodial telomerase will be cloned by screening a genomic *P. falciparum* libraries with oligonucleotide complementary to plasmodial telomeres.

* Studies on plasmodial replication. Electron-microscopic studies will be conducted using decondensed chromatin to determine areas where replication had initiated. In addition, two-dimensional gel electrophoresis as described by Brewer and Fangman will be used to visualize replication intermediates.

* Identification of functional centromere sequences. Centromeric sequences will be identified using a functional, transfection based approach. A wild-type *P. falciparum* strain will be transfected with a *P. falciparum* library cloned in a vector, which contains a selectable marker. Repeated rounds of selection and plasmid recovery, followed by re-transformation, will be employed to enrich for sequences containing a plasmodial centromere. Centromeres will be further dissected by deletion analysis. Protein binding motifs will be tested in gel retardation assays, using nuclear proteins.

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* Spatial and temporal changes of plasmodial chromatin. The chromatin structure of telomeric repeat sequences, subtelomeric regions, internal chromosome domains and the transition between these different domains will be investigated. The possible effect of chromatin changes on the transcriptional activity of developmentally expressed genes will be investigated.
* Characterizing protein components of plasmodial chromatin. Monoclonal antibodies against NAP (Nucleosome Assembly Protein) will be used in immunofluorescence studies to examine the association of this protein with the nuclear material. *In vitro* reconstitution experiments are planned to test the activity of the NAP protein in chromatin assembly.

Expected outcome

⇒ The study aims at characterizing those structural aspects of plasmodial chromatin that are relevant to the multiple functions in which chromatin is involved, such as transcription, replication, segregation, chromosomal stability versus (possibly programmed) rearrangements.

⇒ The study will aid in identifying new targets for rational drug design.

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Contract number IC18*CT970217

DEVELOPMENT OF INHIBITORS OF MALARIAL AND TRYPANOSOMAL DIHYDROFOLATE REDUCTASE AS ANTIPARASITIC AGENTS THROUGH COMBINATORIAL CHEMISTRY

Period: November 1, 1997 - October 31, 1999

Co-ordinator: INSERM, Paris, France (C. DOERIG)

Objectives

- To elucidate the molecular mechanisms regulating sexual differentiation and gametogenesis in the human malaria parasite *Plasmodium falciparum.*
- To identify potential anti-malarial compounds based on chemical inhibitors of protein kinases involved in the regulation of parasite growth and/or development.

Activities

The regulation of *P. falciparum* sexual development requires the tight coordination of several cellular and biochemical processes, such as the control of cell cycle progression and of initiation of DNA synthesis, differential morphogenesis, and stage-specific gene transcription. These processes are likely to be coordinated via signal transduction pathways that are activated by the (as yet unknown) stimuli triggering differentiation. The strategy we will follow to approach this problem will consist of the integration of different lines of investigations:

* Comparativ e biochemical and molecular biology studies using wild-type parasites and mutant parasite lines which have lost the ability to undergo sexual development.
* Identification and characterisation of signal transduction pathways potentially involved in the sexual development of the parasite.
* Study of the interface between the control of initiation of DNA synthesis and upstream signal transduction pathways in the context of gametocytogenesis (cell cycle arrest, inhibition of DNA synthesis) and gametogenesis (sudden release from cell cycle arrest, active DNA synthesis).
* Study of gametocyte-specific transcription and its interface with upstream signal transduction pathways, using the promoter of the early gametocytogenesis marker antigen Pfg25/27 as a model.
* Screening of protein kinase inhibitors as potential antimalarials. Available data on signal transduction and cell cycle control in *P. falciparum* indicate that many genes involved in these processes are conserved in the parasite; these include cyclin-dependant kinases (CDKs), several homologues of which have been identified in *P. falciparum.* The possibility that these enzymes may represent targets for new chemotherapeutic agents will be investigated. The IC50 of several hundred derivatives of CDK chemical inhibitors will be determined on parasite cultures, human cells lines, and *in vitro* kinase assays with purified CDKs from both sources. The purpose of these experiment is to identify compounds inhibiting preferentially the parasite's (versus the host's) CDKs, as the first step towards the development of novel antimalarials.

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Expected outcome

The studies on the molecular mechanisms of Plasmodium differentiation are likely to bring a considerable increase in our understanding of the basic biology of the parasite. It is reasonable to expect that the results of the proposed work will include the identification of components of regulatory networks that are vital to the parasite, and hence will be of significance in the context of novel drug design.

Our attempt to identify parasite-specific CDK inhibitors is an example of the exploitation of results from fundamental studies towards drug development. Although the outcome of such screening approaches is difficult to predict, prospects are promising in the present case, as some of the compounds that will be tested have already been shown to have different IC50s on CDKs from different organisms.

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Contract number TS3*CT920053 and TS3*CT940346

MALARIA PRE-ERYTHROCYTIC STAGES (MPES) EUROPEAN NETWORK ANTIGENS TARGET OF IMMUNE RESPONSES CAPABLE OF INHIBITING *P. FALCIPARUM* PRE-ERYTHROCYTIC DEVELOPMENT

Period: August 1, 1992 - July 31, 1994
January 1, 1995 - December 31, 1997

Co-ordinator: INSTITUT PASTEUR, DEPT. PARASITOLOGIE BIOMEDICALE
Paris, France (P. DRUILHE)

Objectives

- Development of the immunology of MPES with the aim of developing an effective MPES vaccine.
- Acquisition of an improved knowledge of the biology of MPES.
- Improved coordination and exchanges within and between European and Developing Country teams.

Activities

The European malaria pre-erythrocytic stages network is aimed at elucidating the basic biology of, as well as developing control means against, human malaria. It includes:

* Molecular biology studies of pre-erythrocytic antigens from mostly *P. falciparum* and *P. berghei*, and in part *P. reichenowi* (identification, characterisation, production of genes and antigens: LSA1, a major 200 kDa molecule expressed in liver stages; SALSA, a 70 kDa antigen shared between sporozoite and liver stages; LSA3-729, a pre-erythrocytic stage specific molecule expressed in sporozoites and liver stages; DG21, a sporozoite specific 78 kDa molecule). Antigenic features of these molecules, conservation of epitopes amongst isolates, epitope mapping; immunogenicity in animals, characterisation and prevalence of immune responses in humans and in animals (mice and primates), identification amongst the remaining series of cloned pre-erythrocytic stage molecules of those that deserve further detailed studies.
* Improvement of the reproducibility of liver infections in Aotus monkeys. Immunisation, and sporozoites challenges, of chimpanzees and Aotus with *P. falciparum* antigens, of mice and Thammomys with *P. berghei*, *P. yoelii* antigens. Analysis of the immune responses developed by immunised animals and of the type of defence mechanisms operating. Comparison of the type of immunity induced by antigens and by whole parasites (i.e. irradiated sporozoites) in natural versus artificial hosts.
* Analysis of naturally occurring immunity to MPES in field conditions, of the mechanisms regulating parasite loads at the MPES level, and of the main antigens inducing such mechanisms. Analysis of the artificial immunity induced by injection of γ-irradiated sporozoites, and of the mechanisms and antigens responsible for such an immunity.
* Studies of the mode of action and the respective importance of antibodies, antibody-cell cooperation, lymphocyte cytotoxicity and cytokines using *P. falciparum* and human hepatocytes, under *in vitro* conditions or *in vivo* in SCID mice.

Contract number TS3*CT920053 and TS3*CT940346

Expected outcome

Our main goal is the development of an effective means of vaccination. Additionally the project could provide:

⇒ An improved understanding of the human *P. falciparum* relationship at MPES level, mainly through an analysis of existing regulatory mechanisms developed against those stages by exposed individuals and their epidemiological consequences in various areas differing in their vectorial capacity.

⇒ Improved cohesion of European research in epidemiology and diagnostics in concertation with Developing Countries enabling a sustainable research strengthening in malaria endemic areas.

Results (so far)

In general, the malaria pre-erythrocytic stage network has provided a tremendous increase in the output of studies on the molecular biology, immunology and cell biology of human malaria liver stages.

Molecular biology

4 out of 4 of the new *P. falciparum* molecules being studied namely STARP, SALSA, LSA-1, LSA-3 have been characterized in terms of their full length DNA sequence and stage specific expression, and we have initiated the study of three new MPES genes, DG 64, DG 6F and DG 571, two of them in collaboration with Nijmegen and BPRC. Immunological studies with isolates of *P. falciparum* at the sporozoite stage have shown the consistent expression of those genes and comparison of sequence data for areas of immunological interest of LSA-1 and LSA-3, have shown a remarkable degree of conservation, in contrast to many other *P. falciparum* antigens. Homologs of those genes have been found in the ape parasite *P. reichnowi* and for some of them the sequence (e.g. STARP) determined. Immunological and genetic screening of other plasmodial species led to identification of an equivalent of LSA-3 in the rodent species *P. yoelii* which in particular share B- and T-cell epitopes with the *P. falciparum* gene and open the possibility of performing immunization and challenges with the rodent species in laboratory animals.

For immunization purposes, the genes have been cloned in various types of vectors, expressed in prokaryotic, eukaryotic cells and naked DNA vectors. For instance, a very large range of recombinant expression systems are now available for LSA-3 as a model system.

This, together with the large number of lipopeptides derived from the above molecules, provides a very large range of immunization systems.

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Experimental models

One of the strengths of the network lies in the wide range of model systems available:

The primates facilities are unique, particularly in BPRC-TNO, in Cali (Columbia) and in CIRMF (Gabon) by their having chimpanzees and Aotus colonies, the connected excellent research laboratory facilities and the malarial expertise of the corresponding teams.

Chimpanzees at BPRC have been fully MHC-Class-I and Class-II typed. In addition, steps have been taken to develop a novel model in Burma, the gibbon, as an experimental system for erythrocytic and liver stages.

The IMTPL in Belgium has developed a colony of Thamnomys which provided the means to compare host parasite interactions in natural versus artificial hosts. The unique set up in Nijmegen has provided a continuous production of large numbers of *P. falciparum* sporozoites for immunological studies and for challenges. In addition 2 insectaries were developed in Columbia for *P. falciparum* and *P. vivax,* one in IMTPL for *P. berghei* and another one in Paris for *P. yoelii.* Despite the enormous difficulties of this model, the liver stage cultures of *P. falciparum* have been performed on a discontinuous but regular basis in Paris and used to assess *in vitro* defence mechanisms towards *P. falciparum* sporozoite and liver stages.

Many efforts have been devoted to develop a SCID mouse model enabling support *in vivo* for the *P. falciparum* liver stage development in human hepatocytes. Satisfactory progress has been made in this direction since the grafting of functionally active human hepatocytes has been successfully performed and kept for up to four months in this experimental host. However, attempts to infect those hepatocytes *in vitro* have up to now been unsuccessful.

Field set ups

Excellent field facilities have been developed and used for antigenicity studies mostly in Senegal, in Madagascar, but also in the Congo, in Kenya, in Brazil, in Burma and in the Gambia. The contribution of those centres has been invaluable for the identification of epitopes for B-cell, for T-helper cells and for cytolytic T-cells.

In addition, a novel type of field study has been designed and implemented to investigate the existence of naturally occurring anti-IMPES immunity. Some individuals appear to develop a strong resistance to sporozoite challenge and the correspondence between this and the immune responses developed to the molecules under study is expected to provide clues about which molecules induce responses which contribute to protection against MPES.

Contract number TS3*CT920053 and TS3*CT940346

Antigenicity studies

Using peptides and recombinants from the four lead molecules, studies in five endemic areas have identified the sequences defining B-cell epitopes and have showed high to very high sero-prevalences to them (e.g. 97% prevalence to LSA-3 repeats in Senegal spanning all age groups). Similarly T-helper cell epitopes were mapped and a correspondence between the level of malaria transmission and the proportion of T-cell responders was observed (up to 85% T-cell responders to most peptides in the Congo). Many of those epitopes were also found to be able to stimulate the secretion of interferon gamma, the cytokine known to be the most potent in blocking liver stage development. One LSA-3 peptide induces the secretion of particularly high concentrations of this cytokine. Finally, a very large number of HLA class-I restricted CTL epitopes have been identified in the four genes under study: 6 in LSA-1, 2 in SALSA, 1 in STARP and 11 in LSA-3.

Immunogenicity and vaccination studies

Chimpanzee and Thamnomys were used as model systems to analyze the protection induced per irradiated sporozoites and the immune responses in the 18 krad chimpanzee paralleled those recorded in human volunteers immunized in the same manner. However, the CD8 lymphocyte depletion planned in this animal could not be performed. Rodent modelling revealed an inverse relationship between the susceptibility of the host to the parasite and the dose of irradiated sporozoites needed to achieve protection, i.e. protection was very difficult to induce in Thamnomys which is more susceptible than artificial laboratory hosts.

Using the four lead molecules, preliminary immunization attempts were performed in mice of five different H2 haplotypes with many different synthetic peptides and lipopeptides as well as recombinant proteins and a large range of adjuvants. One of the most remarkable results was obtained with lipopeptide which proved able to induce Th and CTL responses without adjuvant and to increase the immunogenicity to the point of apparently overcoming partially or totally the genetic restriction observed in inbred mice. Therefore, immunizations were performed in chimpanzees using six lipopeptides injected in PBS and six non lipopeptides injected in montanide adjuvant, or adsorbed on microspheres. By this means, T-helper cell responses were induced to all twelve peptides, some of them at very high level (stimulation indices > 80), antibody production was observed towards eleven of the twelve peptides, most of them at very high levels.

Moreover, these responses proved to be long lasting and to be specific to parasite native proteins. Finally, CTL responses were detected towards six of those peptides. Challenge studies indicated that SALSA and mostly LSA-3 held the best promise in terms of protection.

A large number of immunization schemes were used in mice with LSA-3 as a model system to assess protection against *P. yoelii* challenge.

Results with QS21, titermax, FCA or FIA were disappointing. To date the best results were obtained with lipopeptide or with microsphere immunization which have the advantage of being effective without adjuvant and to induce a full range of immune responses.

Contract number TS3*CT920053 and TS3*CT940346

In vitro study of defence mechanisms

The *P. falciparum* human hepatocyte *in vitro* system was used to study defence mechanisms. At very low concentration, antibodies to STARP and LSA-3, and also anti-SALSA antibodies strongly inhibited *P. falciparum* sporozoite invasion. IgM from irradiated sporozoite volunteers proved more efficient than IgG antibodies. Several attempts to show whether CTL cells could lyse human or chimpanzee infected hepatocytes have been made so far without reaching a conclusive result. However the conditions necessary to achieve this goal have considerably improved. For instance, a method enabling the raising of CTL malaria specific CTL lines from healthy volunteers, developed in Oxford, increases greatly the chances of matching the MHC Class-I antigen from the effector cells with that of the target hepatocytes. One drug, already developed and used clinically, proved to be very effective *in vitro* upon *P. falciparum* liver stages but not against *P. yoelii* liver stages. G-Oligonucleotide primers derived from LSA-3 were used in a PCR assay which proved to be the most sensitive diagnostics mean available to date to detect very low grade *P. falciparum* blood infection.

In total, these studies have confirmed the potential of the molecules under study, which antigenicity and immunogenicity as well as conservation among isolates appeared to be remarkable. The vaccination schemes employed up to now may not yet be optimal, but have yielded very encouraging results, and the conditions to assay the efficacy of further immunization schemes have greatly improved.

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Contract number TS3*CT920068

MALARIA ASEXUAL BLOOD STAGE EUROPEAN NETWORK (MAS), II

Period: October 1, 1992 - September 30, 1995

Co-ordinator: MAX PLANCK INSTITUT FÜR IMMUNBIOLOGIE
Breigau, Germany (J. LANGHORNE)

Objectives

- The overall objective of this part of the malaria asexual blood stage European Network (MAS) is to acquire some insight into the immune response to the asexual blood stage of malaria parasites with special emphasis on *Plasmodium falciparum*.
- To integrate studies on the analysis of the asexual blood stage antigens MSA-1, MSA-2, PF83 and GLURP.

Activities

The specific activities of this project are:

Year 1

To determine the phenotype and function of T cells from immune individuals before and after stimulation *in vitro* with whole *P. falciparum* infected erythrocytes. 10-20 individuals from each of the following age groups 0-5, 6-10, 11-14, 15-19, 20+ will be monitored in Benin on two separate occasions over the one year period. Isolation and cloning of 10 *P. falciparum* samples from Benin. PCR analysis of variability (MSA1, MSA-2), cataloguing of patients (Malaria-specific antibody responses, different isotypes and specificities).

αβT cell responses of ten immune adults in Benin to SDS-PAGE separated proteins of *P. falciparum*. Basic epidemiological survey of the chosen area in Benin. From the selected immune individuals from ten Benin function of T cells responding to MSA-1, MSA-2, PF83 and GLURP.

Year 2

Further analysis of phenotype and function of responding T cells obtained from Benin. Extension to examination of limited numbers of samples (5 to 10 in different age groups, twice a year) from Mozambique and Zimbabwe. Analysis of variability of cloned isolates (10-20 each from Benin, Mozambique and Zimbabwe).

Analysis of variability of three to five selected *P. falciparum* clones *in vivo* in *Aotus* monkeys.

Further characterization of malaria-specific antibody responses and titre and αβT cell responses of the same immune individuals in Benin compared with 10 individuals in Mozambique and Zimbabwe in cooperation with SSI and TNO.

Contract number TS3*CT920068

Year 3

Further T cell analysis of the remaining samples from all three areas in Africa, evaluation of antibodies and T cells in longitudinal studies. Evaluation of variability of MSA-1, MSA-2, PF84 (in collaboration with TNO) and GLURP (in collaboration with SSI) over the 3 year period in all areas.

Expected outcome

The network activity, possibly with inclusion of new partners, will enhance the understanding of asexual blood stage putative vaccine candidates. The elucidation of the mechanisms operating at the asexual blood stage *P. falciparum* antigens through the analysis of immune-responses developed against the stage by individuals living in endemic areas may further help in the choice of candidate molecules for a vaccine against *P. falciparum.*

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Contract number TS3*CT920116

REGULATION OF SEXUAL DEVELOPMENT IN MALARIA PARASITES AND THE DESIGN OF LOGICAL INTERVENTION STRATEGIES

Period: September 1, 1992 - December 31, 1993

Co-ordinator: IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY & MEDICINE
London, United Kingdom (R.E. SINDEN),

Objectives

Through collaborative studies and associated training programmes, the project aimed to investigate the genetic-, molecular-, and biological regulation of sexual development of *Plasmodium*. Through the information gained logical intervention strategies would be investigated.

Activities

Through mutual exchanges of personnel and reagents between the participating laboratories we have integrated the particular expertise of each laboratory in a series of studies, many of which have been published. The diverse methodologies used have been described in these publications and are therefore not repeated here.

Results

Genetic regulation of sexual development

By comparing the chromosomal location of a large number of genes in different strains of the four rodent species, our data indicated that there is very little gene re-assortment between non-homologous chromosomes. The different chromosomes appear to form stable linkage groups of specific genes in all species. We have initiated a collaboration with the University of Sao Paulo to investigate the genome organization of *P. vivax*. We tested small filters (Plasmodipur; Euro-Diagnostica, The Netherlands) for removal of white blood cells (wbc) from *P. vivax* infected blood. These filters effectively removed wbc while the different developmental stages of the parasites were not trapped in the filters. The older blood stages of *P. vivax* (trophozoites, schizonts and gametocytes) could easily be separated using Nycodenz density gradient centrifugation from uninfected erythrocytes. These parasites have been successfully used for separation of the chromosomes in pulsed field gel electrophoresis.

To elucidate the mechanism(s) responsible for chromosome size polymorphism occurring during mitotic multiplication of *Plasmodium* parasites and the possible correspondence between the appearance of karyotype variants and the loss in the ability to undergo gametocyte differentiation. We characterised a gametocyte-defective clone (HPE) of *P. berghei* which emerged during asexual multiplication of the gametocyte producer clone 8417HP. It exhibits a large subtelomeric deletion of chromosome 5. In *P. falciparum* the effect of a terminal deletion on chromosome 9 (reported to be associated with impaired gametocyte production) on stage specific control of gene expression in sexual differentiation was investigated. Differences in the sexual/asexual pattern of expression of the gametocyte-specific gene Pfg27 were found both at the level of the protein and of mRNA species between the line harbouring the deleted chromosome 9, its parental line 1776 and line 3D7.

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There is evidence that all genes known so far which are involved in sexual differentiation and expressed almost exclusively during and after gametocytogenesis are clustered on chromosome 5 of rodent parasites. These genes are -tubulin-1, Pbs21, C-type rRNA and two other characterized genes.

To provide evidence of the genetic diversity of the "Pbs21 gene" within other rodent malaria species, attempts were made to clone the equivalent gene from different species. The *P. yoelii* equivalent was cloned and sequenced. Screening of two lg11 genomic libraries of *P. vivax* with Pbs21 probes resulted in a number of positive clones between 1 and 6 kb in size but none of these appear to be the Pbs21 homologue.

Molecular regulation of sexual development

To select novel *P. berghei* genes specifically expressed in sexual forms, we devised a subtraction strategy using gametocyte-producer and gametocyte-less clones. A labelled cDNA enriched for sequences selectively expressed in the gametocyte-producer clone 8417HP was obtained after subtraction with a large excess of mRNA prepared from the non-producer clone K173 and used to screen a *P. berghei* genomic library. Among the positive clones, a novel gene which maps to chromosome 5 at a subtelomeric position was selected.

Northern analysis using stage specific RNA preparations from pure cultured sexual forms demonstrated that production of the Pbs21 transcript was initiated in gametocytes yet translation is evident only after gametogenesis and the transcript is considered to be translationally repressed. Preliminary evidence indicates that the start site of transcription lies - 350nt upstream of the translational start site and that processing of the transcript occurs at the 3' end of the mRNA molecule. To find regulatory elements for the expression of Pbs21 two larger cDNA clones (1.1 and 1.25 kb) encoding the Pbs21 gene have been sequenced, 639bp of the region upstream the Pbs21 gene have been described.

In situ detection of mRNA was used to analyze the expression pattern of mRNAs for a number of sexual stage specific transmission-blocking antigens, including Pbs21, Psf25, Pfs28 and Pfs230, throughout gametocytogenesis of *P. berghei* and *P. falciparum*. The initiation of transcription of these mRNAs occurs in a staged series following commitment to sexual development. Pbs21, Pfs25, and Pfs28 mRNAs accumulate in gametocytes in the absence of detectable translation products (collaboration with NIH, Bethesda) suggesting that post-transcription mechanisms operate to regulate the translation of the protein (see above).

We have developed probes which allow the demonstration of transcriptional activity of the two classes of rRNA gene in the highly developed model available in the laboratory. The probes detect the external transcribed spacer (ETS) of the two types of rRNA unit and demonstrate not only transcription but also the degree of conservation between the genes comprising the two types of rDNA unit.

To identify cdc2 products in *P. berghei* extracts, immunoblot assays were conducted using a commercial monoclonal antibody directed against the highly conserved domain PSTAIRE. A protein of 30kDa was detected in young trophozoites only. We were able to amplify a 1 kb fragment of a gene, showing a high homology to cdc2, in 5 *Plasmodium*-species: *P. knowlesi, P. berghei, P. vinckei, P. chabaudi and P. yoelii.*

These fragments were cloned and sequenced. In cooperation with the University of Sao Paulo, Brazil, the same fragment of the *P. vivax* gene was also cloned and its DNA-sequence determined. In the 1 kb fragment of all species under study, three introns are present at conserved loci. By comparing these introns with respect to a number of characteristics, we were able to draw some conclusions on intron-organisation and evolution within the genus *Plasmodium*.

Biology of sexual regulation

Mechanisms of transmission blockade in infected hosts. The natural decline of infectivity of gametocytes three days post infection is not antibody mediated since it could be demonstrated that the pattern of infectivity was exactly the same in severe combined immunodeficient mice (scid) as in their intact Balb/c congenic partners. The resulting inhibition of the parasites' sexual cycle within the mosquito occurs within one hour of the mosquito feed. To date, there is no significant evidence that nitric oxide or its derivatives are involved in the blockade of either *P. berghei* or *P. vinckei* infections.

Studies were carried out to test cryopreservation efficacy of sexual stages of *P. vivax* malaria parasite from blood from patients. Zygotes were obtained *in vitro* and unfrozen and cryopreserved blood infectivity was tested by *An. albimanus* infections. The proportion or recovery was similar to that obtained in cryopreserved asexual stages of the parasite.

Development of logical intervention strategies

Studies on the expression and immunogenicity of recombinant Pbs21 expressed in a baculovirus system revealed that:

⇒ Expression of the recombinant protein in insect larvae results in higher yields than expression in *in vitro* systems.
⇒ The protein is indistinguishable from native protein by means of conformation-dependent antibodies.
⇒ Deletion of the putative signal sequence prevented protein expression on the cell surface.
⇒ Transmission-blocking activity induced by the full length protein in mice was higher than 90%.

We have evidence that the protein has either a novel type of GPI anchor or is an acylated membrane protein. Neither PIPLC treatment nor nitrous acid deamination had any effect on the molecule but with hydroxylamine treatment cleavage of the membrane anchor was obtained.

Infected blood erythrocytes obtained from *P. vivax*-infected patients, in their original plasma or plasma obtained from uninfected normal donors, were offered to *An. albimanus* and transmission blocking activity was estimated by comparison of the infection rates obtained with the 2 preparations. Protein extracts were prepared from purified *P. vivax* gametocytes and used for immunoblot assays of sera with transmission blocking activity. Several protein bands were identified. The most frequently observed were protein bands of 113, 103, 94, 85, 68, 47, 41, 37 and 31 Kda.

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MALARIA ASEXUAL BLOOD STAGE EUROPEAN NETWORK (MAS), I

Period: October 1, 1992 — September 30, 1995

Co-ordinator: STATENS SERUM INSTITUT, DEPT OF PARASITOLOGY, Copenhagen, Denmark (B. HÖGH)

Objectives

- The overall objective of the malaria asexual blood stage European Network (MAS) is to acquire a comprehensive insight in some of the basic questions of the biology of the asexual blood stage of malaria parasites with special emphasis on *Plasmodium falciparum.*
- To integrate studies on the analysis of the asexual blood stage antigens MSA-1, MSA-2, Pf83 and GLURP.

Activities

The specific activities of this project are:

Year 1

Production of prokaryote expressed GLURP and GLURP fragments in *E. coli,* and eucaryote expression of the entire GLURP antigen in vaccinia virus. Map potential protective immunogenic epitopes of GLURP in mice and rabbits. Assess the protective immunogenicity of GLURP in *Aotus* monkeys. Immunisations will follow the current protocol at WRAIR. An evaluation of GLURP produced in *E. coli* and encapsulated in liposomes with lipid A, and GLURP recombinant vaccinia virus will be performed by SSI in collaboration with WRAIR.

Examine the expression of GLURP in liverstage parasites. Anti-GLURP peptide sera will be used for IFAT and immunoelectron microscopy of cryosections of liverstage parasites taken from chimpanzees infected with *P. falciparum* will be performed by SSI in collaboration with TNO.

Prepare for field studies in Mozambique and Zimbabwe, collect baseline parasitological and entomological data and establish techniques for field studies: ELISA, PCR etc. for both molecular biological and immunological studies will be performed by SSI in collaboration with TNO and MPI (begin T cell analysis, spot ELISA for cytokine production and PBL phenotype before and after stimulation with antigens, PCR analysis of antigen where necessary).

Year 2

Continue the activities initiated during year one. Perform field studies — parasite immunology and entomology in Mozambique and Zimbabwe, with established techniques performed by local researchers in collaboration with SSI, TNO and MPI.

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Year 3

To correlate human immune responses to GLURP with exposure, age and disease in longitudinal epidemiology studies performed by local researchers in Mozambique and Zimbabwe in collaboration with SSI, TNO and MPI. Assess the conservation of the putative protective immunogenic epitopes of GLURP.

Rounding off the field and laboratory work. Statistical evaluation and presentation of the results in relevant peer-reviewed journals. Planning new research.

Expected outcome

It is hoped that the network activity, possibly with inclusion of new partners, will enhance the understanding of asexual blood stage putative vaccine candidates. The elucidation of the mechanisms operating at the asexual blood stage *P. falciparum* antigens through the analysis of immune responses developed against the stage by individuals living in endemic areas may further help in the choice of candidate molecules for a vaccine against *P. falciparum*.

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MALARIA ASEXUAL BLOOD STAGE EUROPEAN NETWORK (MAS), III

Period: February 1, 1993 - January 31, 1996

Co-ordinator: BIOMEDICAL PRIMATE RESEARCH CENTRE BPRC,
DEPT. OF PARASITOLOGY,
Rijkswijk, The Netherlands (A. THOMAS)

Objectives

- The overall objective of the malaria asexual blod stage European Network (MAS) is to acquire a comprehensive insight into some of the basic questions of the biology of the asexual blood stage of malaria parasites with special emphasis on *Plasmodium falciparum*.

- To integrate studies on the analysis of the asexual blood stage antigens MSP-1, MSP-2, PF83/AMA-1 and GLURP.

Activities

Year 1

Express full length recombinant PF83/AMA-1 and clone and express full length recombinant PV66/AMA-1, and develop mid/large scale expression cultures. Begin rodent PF83/AMA-1 immunisation for cross protection studies. Assess non-immune and immune adult serum reactivities with PF83/AMA-1 to establish baseline values.

Year 2

Purify PF83/AMA-1 from expression cultures in amounts sufficient for 1) seroepidemiological study, 2) for T-cell lymphoproliferative assay in endemic areas and for *Aotus* immunisation trial. Develop a similar strategy for PV66/AMA-1 and develop truncated constructs for both PF83 and PV66 that may be expressed at higher levels. Begin mAb production vs AMA-1 constructs. Assess naturally occuring variation in PF83 and analyse *P. reichenowi* AMA-1 sequence.

Year 3

Continue biochemical and immunological characterization of AMA-1 molecular family. Analyse mAb activity against parasite development. Determine utility of *Pichia pastoris* expression for PV66.

Contract number TS3*CT920147

Results

The European network for asexual blood stage vaccine development has followed an integrated approach and has benefited significantly from the inclusion of enthusiastic and productive developing country partnerships. Within this contract diverse approaches have been followed to develop an understanding of AMA-1 as a vaccine candidate molecule. The work has reinforced the candidacy of the *P. falciparum* 83 kDa AMA-1 molecule as a target for parasite inhibitory responses, by showing that despite the difference in molecular weight, both 83 and 66 kDa forms of AMA-1 are similarly targeted by antibodies that block parasite multiplication. Purified recombinant PF83/AMA-1 was highly immunogenic, and induced parasite inhibitory responses.

One of the most important findings was that a new yeast expression system, *Pichia pastoris*, is ideally suited to very high level expression of the *P. vivax* form of AMA-1, and that such material is correctly folded and immunogenic. Discussions surrounding the generation of clinical grade PV66 have been initiated. The prospects for *P. falciparum* AMA-1 expression in this efficient system are also promising. A transcriptionally active block to *P. falciparum* AMA-1 expression has been identified and the gene mutagenised to try to overcome the problem.

Because AMA-1 is so highly constrained in its conformation, any deviation from a "native" type structure may have significant negative consequences. All eucaryotic expression systems are likely to glycosylate, often very heavily, at sites within the gene that are not normally glycosylated. All potential N-glycosylation sites were identified, and conservatively removed by mutagenesis (AMA-1 does not appear to be glycosylated in the parasite). This has been completed for *P. falciparum*, *P. vivax* and *P. knowlesi* AMA-1 molecules. As a result a highly homogeneous product is now produced in the expression systems. The folding of the mutagenised recombinant AMA-1 molecules is equivalent to the "native" form of the molecule. Similar levels of PV66 expression were obtained for both molecular forms, and comparative studies are underway.

Initial work with a naked DNA system, using mutagenised AMA-1 variants, suggest that expression of AMA-1 can be achieved. Given the immunogenicity of AMA-1 that we have also shown, it suggests that even at low level expression DNA vaccination may be of great value for AMA-1.

Using the most effective recombinant systems that we have so far identified for *P. falciparum* AMA-1 and *P. vivax* AMA-1 (baculovirus and yeast respectively), we have devised purification regimes to provide material for a wide range of studies. AMA-1 has been prepared for ELISA (and made available to many groups worldwide), for T-cell studies and for immunisation studies.

Evidence that *P. falciparum* AMA-1 could be functionally blocked came from studies on mAbs to the molecule. Two mAbs were identified that inhibited red cell invasion by the merozoite. Sera from endemic regions contained antibody specificities that strongly competed with the binding of these mAbs to AMA-1 immunisation of rats and mice induced

polyclonal antibodies that also blocked parasite multiplication. During the immunisation studies a variety of adjuvants and immunising doses were compared. There was clear evidence that even without adjuvant the molecule was higlhy immunogenic.

An immunisation study using an Aotus monkey challenge model has been completed and is being evaluated.

One of the most intriguing studies has analysed the molecular fate of AMA-1. This has produced the first evidence for malaria parasites of a processing dependant re-localisation of antigen. Furthermore, the molecular characteristics of AMA-1, and the timing of the processing serve to link the molecule intimately with red cell invasion.

Contract number TS3*CT920147

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Contract number TS3*CT930228

NATURAL AND ARTIFICIALLY INDUCED IMMUNITY AGAINST PLASMODIUM LIVER STAGES

Period: September 1, 1993 - August 31, 1996

Co-ordinator: GROUPE HOSPITALIER PITIE-SALPETRIERE, Paris, France (D. MAZIER)

Objectives

- To analyze the immune response induced by "known" multi-stage epitopes expressed during the hepatic phase of malaria parasite development.
- To identify "new" epitopes expressed on the surface of infected hepatocytes.
- To gain an insight into immune regulation between the hepatic and the other stages of infection, with an emphasis on the development of cerebral malaria.

Activities

1. Studies will concern:

* T-cell epitopes of the non-repetitive part of the CS protein (*P. falciparum, P. yoelii* and *P. berghei*).
* Heat-shock like epitopes (*P. falciparum*).
* Merozoite surface proteins MSP1 (*P. falciparum* and *P. yoelii*).

Work will be focused on:

* MHC-restricted cytotoxicity mediated by CD4+ and CD8+ T cells.
* Antibody dependent cellular cytotoxicity (ADCC).

Immunizations will be performed using the epitopes alone or coupled together in Multiple Antigen Peptide systems (MAPs). *In vitro* studies carried out in parallel with those *in vivo* will utilize hepatocytes and other liver cells from the same host in order to make comparisons as valid as possible between both *in vivo* and *in vitro* infections, and the human and animal studies in culture.

2. The natural peptide fragments of *P. falciparum*, *P. vivax* and *P. yoelii* antigens will be isolated by acid extraction and extracts will be fractionated by reverse-phase HPLC.

Individual peptide fractions will be tested:

* For their ability to react with sera from immunized and protected animals or with sera from humans living in endemic areas.
* For their ability to stimulate splenic T cells obtained from the same animals or peripheral T lymphocytes from humans.

In positive fractions, peptide structure will be determined by microsequencing. Peptides will then be synthesized. DNA probes will be synthesized from the primary structure of isolated peptides, amplified by the PCR method and the genes coding for these proteins localized in a DNA library. The peptides will then be analyzed for their immunogenicity and their potential role in protection.

3. We shall investigate whether interference with hepatic stage development modulates subsequent cerebral malaria complications.

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The role of cytokine regulation will be analyzed in humans in endemic areas by following changes in cytokine profiles and pattern of reinfection. We shall interpret these data using rodent malaria infections in the natural hosts and in mice with different haplotypes and backgrounds.

Expected outcome

⇒ A better knowledge of the plasmodial antigens that could induce protection against the hepatic stages.
⇒ A better understanding of the host-parasite interaction, specially the parasite mechanisms of escape from the immune response, to aid in development of a vaccine.

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Contract number TS3*CT930229

REGULATION OF SEXUAL DEVELOPMENT IN MALARIAL PARASITES AND THE DESIGN OF LOGICAL INTERVENTION STRATEGIES

Period: January 1, 1994 - March 31, 1996

Co-ordinator: IMPERIAL COLLEGE OF SCIENCES, TECHNOLOGY & MEDICINE, DEPT. OF BIOLOGY, London, United Kingdom (R. SINDEN)

Objectives

Through collaborative studies and associated training programmes, the project aimed to investigate the genetic, molecular and biological regulation of sexual development of *Plasmodium*. Though the information gained logical intervention strategies would be investigated.

Activities

Through mutual exchanges of personnel and reagents between the participating laboratories we have integrated the particular expertise of each laboratory in a series of studies, many of which have been published. The diverse methodologies used have been described in these publications and are therefore not repeated here.

Results

Expression and immunogenicity of recombinant transmission-blocking antigens

A preliminary comparative study assessing the immunogenicity of full length Pbs21 and Pbs21 lacking the anchor region has shown that antibodies reactive to native Pbs21 were induced in both groups of immunized mice, however pronounced differences in the titre of antibody responses in Western blot analyses and in the transmission-blocking activity of immune sera were observed.

Three recombinant baculovirus containing the full length coding region of Pfs28, the *P. falciparum* homologue of Pbs21, were purified and patterns of protein expression in insect cells was determined by Western blot analysis, and optimal conditions for antigen production were determined. The immunogenicity of the recombinant protein will be tested in immunization experiments and compared to recombinant Pfs28 expressed in yeast (obtained from NIH, Bethesda).

A plasmid for DNA vaccination containing the Pfs28 gene was constructed. Plasmid mediated protein expression will be tested using a mammalian cell system.

A novel *P. berghei* sexual stage specific protein was identified. Native protein was purified by electroelution. Preliminary data suggest that the protein is expressed in zygotes and ookinetes starting approximately 6 h after activation of gametocytes. Immune sera against the protein and monoclonal antibodies are currently being developed to further characterize the protein and to screen an ookinete specific cDNA library.

rRNA regulation

In situ hybridization confocal laser scanning microscope studies on the regulation of rRNA during sexual development are described elsewhere in the report.

The identification of proteins that are phosphorylated/dephosphorylated.

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The phosphorylating and H1 kinase activities of extract preparations of *P. chabaudi* and *P. berghei* parasites were analyzed along the erythrocytic cycle. In experiments using *P. berghei* extracts, histone was intensively labelled in young trophozoite extracts; this activity decreased in mature trophozoites and disappeared in schizonts. Labelling of non-histone proteins was parasite stage specific: a band of >200 kDa was phosphorylated in young trophozoites, a band of 45kDa was labelled in mature trophozoites and a 40 kDa protein was phosphorylated in schizonts. Quantitative analysis of ^{32}P-ATP incorporation was carried out in *P. chabaudi.* Major histone kinase activity occurs in mature trophozoites. In the absence of histone, no incorporation of ^{32}P- was detected in schizonts. When H1 histone was added, stimulation of the phosphorylation occurred in all asexual parasite stage extracts. Experiments to study phosphorylation in *P. berghei* sexual stages have produced contradictory results mainly because of asexual stage contaminations. We have standardized purification techniques for micro- and macro- gametes, zygotes and ookinetes and are currently using these preparations.

cdc-2 like kinase activity; and expression of cyclin during the different phases of the parasite life cycle. The peptide sequence reported for a highly conserved region of the cyclin molecule of several species, between positions 201 and 335, named the "cyclin box" and the codon usage of *P. falciparum* were used for PCR-amplification of *P. chabaudi* DNA. The products obtained were cloned in the Small site of pBluescript plasmid for their characterization. The sequencing and analysis of the inserts obtained are in progress. Preliminary comparisons of the 400 bp band indicated sequence homology with human G protein coupled receptor; human gene 1AC; and several anonymous sequences of the *P. falciparum* genome (including genomic clone 0433). The amino acid sequence shared homology with yeast cell division control protein 10 (20% in a 38 aa strand); mouse tyrosine receptor FLT4 (66% in a 10 aa strand), a putative serine/threonine kinase R107.4 (83% in a 6 aa strand) and a similar degree of homology (40% in 18 aa strands) with zinc fingers of rat, mouse and human. We are currently sequencing the other clones.

Molecular karyotype changes related to gametocytogenesis in P. berghei and in P. falciparum

In *P. berghei* several sexual-specific genes have been characterized, which map to chromosome 5. Moreover, rearrangements which affect this chromosome correlate with defects in the gametocytogenesis. Its structural organization has been studied in detail in collaboration with the University of Leiden. A long-range map of chromosome 5 from the gametocyte-producer clone 8417HP, taken as a reference clone, has been constructed and genes hybridizing to it positioned. Subtelomeric portions of this chromosome have been studied in more detail. They are characterized by the presence of a region, extending 60 kb at most, shared by both the extremities and symmetrically located. This region is involved is most large-scale rearrangements affecting this chromosome.

In *P. falciparum* the role of the terminal portion of chromosome 9 in gametocyte differentiation has been investigated. By analyzing a synchronous parasite population of line HB3, heterogeneous for the size of chromosome 9, it was shown that the small fraction of full-length chromosome 9 was preferentially retained in those parasites developing into gametocytes. Studies on isogeneic parasite lines (derived from isolate 1776) differing for the size of chromosome 9 demonstrated that parasites harboring the deleted forms do not enter the earliest detectable step of sexual differentiation. This suggests a role of functions encoded in that region of chromosome 9 in the regulation of early events of sexual differentiation.

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Regulation of sexual/asexual gene expression

A novel gene family, which maps to the terminal portions of *P. berghei* chromosome 5 (one member was selected by subtraction procedures), has been characterized. It contains three partially homologous genes which share the N-terminus of the deduced proteins.

The genomic region of one of these three genes is involved in large scale subtelomeric rearrangements observed in two characterized gametocyte-defective mutants.

A *P. berghei* gene (pbB7) conserved within the Plasmodium genus is also being characterized The coding sequence exhibits significant blocks of similarity with a class of nucleosome assembly proteins. The nuclear localization of pbB7 gene product has been demonstrated both in *P. berghei* and *P. falciparum* using specific monoclonal antibodies. A significant difference in the size of the corresponding transcripts has been observed when comparing sexual and asexual parasites. Introns have been detected positively and a possible mechanism of alternative splicing is under investigation.

In order to describe the promoter of Pfg27 gene of *P. falciparum* and its regulation, a structural and functional analysis was undertaken on the genomic region upstream the gene which is active in early stages of gametocytogenesis. Northern blot analysis, RNAse protection and nuclear "run-off" experiments on sexual and asexual stages permits us to state that the expression of this gene occurs at the level of transcription initiation. The gene is transcribed in the first 2 to 3 days of sexual differentiation, while it stops afterward.

Genetic transformation

This work has included:

⇒ The successful development of a drug selectable system for the genetic transformation of the rodent malaria parasite, *Plasmodium berghei*, based upon the donation of drug resistance to the antimalarial drug pyrimethamine. A copy of the homologous dhrf/ts gene containing a Ser/Asn110 mutation has been engineered into *E. coli* plasmids creating transfection vectors. A series of vectors have been constructed that are designed to integrate in a site specific fashion into the parasite genome.

⇒ The use of the system to transform and select transformed parasites that maintained the vectors as episomes. The further characterization of the biological properties of the plasmid DNA maintained in the transformed parasites.

⇒ The successful and site specific introduction of foreign DNA into subtelomeric regions of three *P. berghei* chromosomes demonstrating that subtelomeric structures can support expression of RNA polymerase II transcribed genes.

Gene mapping

Collaborative studies revealed that the location and linkage of genes on chromosomes of rodent malaria parasites are highly conserved. The link between chromosome 5 and sexual development has been investigated. In different non-gametocyte producing parasite clones a specific rearrangement in the subtelomeric regions of this chromosome has been observed and genes involved in sexual development appear to cluster on this chromosome. In one of our collaborations a long range restriction map of the chromosome has been produced and the size reduction shown to consistently involve rearrangements in a single region of the chromosome. A YAC library of a gametocyte producing parasite clone has been produced and to date YACs covering 60% of chromosome 5 have been isolated.

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Gene expression

The cloning and mapping of the 5' regions of model genes has resulted in identification of the promoter regions of both A-type rRNA genes and the Pbs21 gene. The latter was shown by *in situ* mRNA hybridization on bloodstage parasites to be transcribed only in female gametocytes. Transient transfection technology for the functional analysis of the structure of these promoters is under development.

The precise pattern of expression of the stage specific rRNA genes has been determined throughout most of the *P. berghei* life cycle (collaborations with Imperial College).

In collaboration with University of Sao Paulo, the cell cycle gene crk2 has been cloned and characterized from a number of different malaria species (*P. berghei, P. knowlesi* and *P. vivax*) as have the genes encoding the ribosome associated EF-1 alpha protein. The genetic and biochemical characterization of crk2 is in hand. This collaboration also initiated studies on the organization of the genome of *P. vivax* involving a 6 week visit of a student from USP.

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Contract number TS3*CT940272

STUDIES ON HUMORAL AND CELLULAR IMMUNE RESPONSES IN HUMANS TO PREVIOUSLY DEFINED MALARIA VACCINE CANDIDATES

Period: August 1, 1994 - July 31, 1997

Co-ordinator: INSTITUT PASTEUR, Paris, France (L. PEREIRA DA SILVA)

Objectives

Identification of B and T cell epitopes present in recombinant *P falciparum* antigens recognised by the human immune system in relation to naturally acquired protective immunity; search for human genetic factors (in particular HLA) involved in the development of immunity to malaria infection.

Activities and Results

Field studies on anti-disease and anti-parasite premunition

Our analysis of the protective role of parasite antigens is based essentially on the comparative studies of immune responses in protected and susceptible individuals exposed to malaria infection. This is performed by a longitudinal clinical-parasitological survey with a permanent follow-up of human populations from endemic areas of Senegal and Brazil: a) inhabitants of two villages (Dielmo and N'Diop) in the holo- and hyper-endemic area in Senegal and b) the Candeias, Urupa and Porto Chuelo sites in Rondonia, Brazil (hypoendemic malaria with epidemic episodes).

Previous studies from various research groups (including our own) have been done using the criteria of age to define the development of premunition. However, the field studies developed by our groups in Senegal and Brazil, in the last year show the limitation of these simplified criteria. The following recent results illustrate these limitations and make a case for a more precise definition of premunition.

Our longitudinal survey allows a comparative clinical and parasitological analysis of the populations from N'Diop and Dielmo villages, which are situated only 5 km apart in the Side Saloum area of Senegal. The presence of a permanent stream running through Dielmo provides permanent breeding sites for *Anopheles* mosquitoes and an intense and perennial malaria transmission (around 200 infective bites per person per year). In N'Diop transmission occurs intensively only in the rain season (around 20 infective bites per year concentrated in the four months rainy period).

The annual incidence of malaria attacks as well as parasite index differs considerably in both villages: adults from N'Diop present a higher number of malaria attacks; children

under 5 years old from Dielmo present twice as many malaria attacks than children from N'Diop. However, in N'Diop children over 5 continue to present a high frequency of malaria attacks until they are 12 - 14 years old.

Another interesting observation concerns the evolution of clinical immunity: it is accepted that premunition is characterised by a decrease in the number of malaria attacks. We tried to verify if this age dependent immunity corresponds also to a decrease in intensity of symptoms in immune adults. However, detailed studies of symptoms and quantified signs (temperature, sudoresis, vomiting) indicates that the only clear difference is found in the duration of the symptoms.

Other observations concern the non-specificity of clinical immunity conferred by malaria parasites. In Dielmo it is observed that clinical attacks by *P malariae* are quite rare in spite of the high prevalence of parasites of this species in the blood of children. In Rondonia, Brazil, where clear premunition was not observed in various cross-sectional surveys. Interference between *P falciparum* and *P vivax* infections is also observed.

In the light of these and other observations, the immune status (level of clinical and anti-parasite immunity) is now defined individually, in relation to the evolution of the infection in the child or in the adult (asymptomatic or symptomatic; stable or unstable parasitemia) in the periods preceding and succeeding the time when samples of sera and/or cells are taken for analysis.

HLA typing

Following previous analysis of HLA, class I antigens typing of Dielmo habitants we have performed analysis of HLA-A, B, C, DR and DQ in 116 habitants of Dielmo from the Serere ethnic groups. No statistically significant differences were observed in the frequency or distribution of the 25 different alleles identified in the Dielmo Serere in relation to the results described by other authors concerning the Mandinka groups from Senegal and Serere and the Mandinka groups of the Gambia.

Immunological studies in the endemic areas

In the last year we have concentrated our studies in Dielmo on characterisation of the isotope specific antibody responses against total and specific *P falciparum* antigens.

This was justified by previous results showing the protective antibodies are not neutralising antibodies, but are cytophilic antibodies (bind to Fc receptors of macrophages). The anti-parasite activity depending on mechanisms of ADCI and/or opsonization/phagocytosis.

Serum samples from three cross-sectional surveys in the Dielmo village (145 habitants of all age groups) were used for analysis of antibody isotypes. In a first approach, total antigen of *P falciparum* was used for measuring total anti-malarial antibodies of the IgM and IgG class and of IgG sub-classes in a ELISA assay. Adults had higher levels of specific antibodies than children. With IgM, IgG2 and IgG3 accounting for the difference.

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Differences in antibody levels were significant for IgG1, IgG2, IgG3 and IgG4 between the lowest and the highest transmission seasons (while infective bites/person/night increased around 20 fold). No particular isotype distribution pattern could be found to be associated with any given parasitemia level. The relationship between the OD values of each isotype and the risk of clinical malaria attack (in the period following the serum sampling was tested using a Poisson regression model. Only the IgG3 OD increases were found to be associated with a significant reduced risk of malaria attack. These seroepidemiological data suggest that, whereas the total IgG specific activity is not indicative of any given level of protection against malaria, the level of IgG3 was significantly associated with the relative susceptibility to clinical malaria attacks. The analysis of antigen specific IgG3 levels is underway. Preliminary data indicate an increase in anti R45 and anti MSP-3 antibodies of the IgG3 isotope in individuals with reduced risk of malaria attacks. Studies are also in progress on the measurement of antibodies against the different fragments of the C terminal part of the MSP-1 antigens concerning the 42 Kd and 19 Kd processing of products. MSP-1isolated and characterised by the MRC laboratory.

The Rondonia samples show an increase in the level of antibodies against EB200 (Pf332) and Pf72 antigens as a function of age and exposure to malaria infection. However, no correlation could be observed with any premunition. Isotype analyses are now in progress.

Contract number TS3*CT940272

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Contract number TS3*CT940317

IDENTIFICATION OF MALARIA VACCINE CANDIDATES BY PASSIVE TRANSFER IN INFECTED INDIVIDUALS OF ANTIBODIES WITH DEFINED SPECIFICITIES

Period: November 1, 1994 - October 31, 1997

Co-ordinator: INSTITUT PASTEUR, DEPT. PARASITOLOGIE BIOMEDICALE
Paris, France (P. DRUILHE)

Objectives

We propose here a novel approach to vaccine design based on the study of human-parasite interaction both for the selection of molecules and the evaluation of results, that is an approach in which the clinical and parasitological response of infected humans is the main screen.

Our objectives are:

- To identify the target antigens of acquired immunity ("premunition") for vaccination purposes.
- To produce and evaluate therapeutic antibodies.

Activities

* Isolation of antibodies with well-defined specificities by affinity-purification of human immune IgG on relevant peptides and assessment of their *in vivo* efficiency by passive transfer in recrudescent asymptomatic *P. falciparum* cases.
* Further epidemiological studies by means of *in vitro* cell-antibody cooperation assays (ADCI) in order:
  - to confirm the relevance of the target antigens already selected and to identify cross-reactive epitopes, if any;
  - to define if there are other antigens targeted by host defense mechanisms.
* Development of an immuno-deficient mouse model receptive to human malaria in order to further our understanding of host defense mechanisms and relevant target antigens.
* Development of the technology of recombinant human antibodies specific to *P. falciparum* antigens as a means of producing large amounts of identical antibody specificity(ies) which may prove to be effective by passive transfer and which could be used for clinical treatment.

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Expected outcome

The present project is expected to supply a novel means of assessing new vaccine candidates in order to speed up the development of a malaria vaccine.

In view of the lack of a model established as being relevant to the human-*P.falciparum* immune relationship, this approach to vaccine design is based on the study of human-parasite interaction both for the selection of molecules and the evaluation of results, is therefore clinical and parasitological responses of infected humans are the main screen.

Results

The first objective has progressed according to plan

⇒ The relevance of MSP-3 as a target of protective antibodies was confirmed by means of ADCI studies.

⇒ In addition, we have now identified two additional targets of the Monocyte-dependent Antibody-mediated inhibitory mechanism (ADCI). One of them is the GLURP molecule expressed at the merozoite surface level in which not less than five B-cell epitopes were identified; affinity purified antibodies were produced and each of the epitope specific antibodies proved able to exert an *in vitro* inhibition of *P. falciparum* growth in ADCI assay. Another target is the SERP molecule which is studied in collaboration with the groups of D. Camus, in Lille, and of J. Inselburg in the USA.

⇒ Previous indications of potentially cross reactive epitopes between two of the above three molecules were not confirmed by further investigations.

⇒ A potential fourth target of ADCI has been identified in MSP-1 through a collaboration with Dr. A. Holder and E. Riley. One EGF domain would be the target of ADCI type of defence without direct effect of antibodies, and the second EGF domain would be the target of a direct effect of antibodies. However, the initial results are only indicative and deserve to be re-evaluated with a novel batch of human IgG which has been recently prepared (see below).

⇒ Considerable efforts have been devoted to the development of an improved and reliable *P. falciparum*-SCID mouse model. This model has first of all a potentially very large number of applications in the field of drug and vaccine design. Moreover, it becomes more important than previously in view of the severe delays that the novel French legislation for blood derived products has imposed on a part of our project (see below B). Compared to the initial protocol, we have introduced a number of modifications to the scheme of additional immunodulation of the mouse immune system.

⇒ These methods have enabled us to reach higher parasitemia up to 38% of infected RBCs. However, we are still encountering a lack of reproductibility in the results, a

limitation to more extensive use of the model. We are therefore presently concentrating our work on means to improve the reproducibility. Nevertheless, preliminary passive transfer experiments could be performed using whole human African IgG from protected individuals and showed a total absence of direct effect of clinically effective antibodies. Conversely, we have gathered evidence, though it now needs to be confirmed, that the same antibodies become effective when mice are grafted with human monocytes.

⇒ We have also performed systematic studies on the best technical conditions to perform on a large scale, and ultimately in GMP conditions, the affinity purification of defined antibodies extracted from whole African IgG, upon insoluble peptide covalently coupled to a matrix. One of the difficulties is that the antibodies of highest affinity cannot be detached from the affinity column without denaturation. However, through a systematic study of various releasing agents, one ensuring the best compromise, has been identified for MSP-3.

The second objective contains 2 distinct parts

⇒ The *in vivo* study of the clinical effect of defined antibodies is suffering considerable delay due to the major changes which were made to blood derived products regulations following the haemophilus scandal and Ebola virus epidemic. This is understandable, but induced unexpected delays in the handling of plasma of African origin by Western transfusion centers. Nevertheless progress could be made so that the new pool of African malaria-immune plasma is now ready, and that fractionation at bench level, but in the same conditions used for GMP preparation, can now be made and will serve at least for pre-clinical steps (e.g. ADCI and SCID work).

⇒ The other part about recombinant antibodies carried on in collaboration with Dr. Dziegel has progressed well. Lymphocytes RNAs were successfully extracted from two pools of twenty African donors living in a hyperendemic area of Senegal. The yield and integrity of RNA was satisfactory and allowed the successful preparation of two cDNA libraries. From the first library, the screening of malaria specific recombinant antibodies was first performed using GLURP antigen by panning of filamentous phages and led to identification positive clones. The specificity of these antibodies, first demonstrated by ELISA, was confirmed by Western-Blotting as well as by immunofluorescence studies. Further screening of the libraries with MSP-3 antigens is now planned.

Contract number TS3*CT940317

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Contract number TS3*CT940345

ASSESSMENT OF IMMUNE RESPONSES INDUCED IN PRIMATES IMMUNIZED WITH LIPOPEPTIDES DERIVED FROM *P. FALCIPARUM* MPES ANTIGENS

Period: January 1, 1995 - December 31, 1998

Co-ordinator: INSTITUT PASTEUR, DEPARTEMENT PARASITOLOGIE BIOMEDICALE
Paris, France (P. DRUILHE)

Objectives

- To construct single and multiple epitope peptides containing a lipid moiety using the sequences already identified in LSA1, LSA3, SALSA and STARP molecules.
- To analyze the immune response induced in primates by such peptides.

Activities

The immune responses induced by lipopeptides in mice, chimpanzee and *Aotus* will be analyzed in order, firstly to detect B, T and CTL responses and secondly to evaluate the possible importance of CTL in defence against human malaria liver stages.

Results

⇒ Additional epitopes have been identified by elution of self-peptides from defined HLA antigens namely: A2, B8, B27, BW53, B35, by further determination of amino-acids sequences of pooled peptides and identification of critical binding residues, by identification in the sequence of MPES antigens of octamers and nonamers bearing the same motif, by synthesis and testing of these peptides in an HLA-assembly assay, and finally by CTL assays performed in malaria-exposed populations, for instance 11 CTL epitopes were identified in the LSA3 antigen.

⇒ Larger peptides (16-34 AA) including a lipid moiety (Palmitoyl-Lysine) introduced using a simple and single step procedure have been synthesized on the basis of the above findings, or of predictions based on molecular analysis (i.e. flexible junction areas) for antigenic analysis and mostly for immunization purposes.

⇒ The lipo-peptide LSA3-NR II, of 26 amino acids, derived from the *Plasmodium falciparum* LSA3 antigen was first employed for immunisation of mice and chimpanzees.

Comparative immunisation by this peptide with and without the lipidic tail shows that:

⇒ The C-terminal addition of a palmitoyl chain can dramatically increase Th cell responses in a wide range of mice MHC Class-II haplotypes, to the extent that strong responses were induced in animals otherwise unable to respond to the peptide by classical immunization, (i.e. with Freund's adjuvant);

⇒ The increased immunogenicity of the lipopeptide led to high and long lasting antibody production without the need to use powerful adjuvants, i.e. with a formulation that can be used in humans;

⇒ B and T-cell responses induced by the lipopeptide were reactive with native protein epitopes;

- large lipopeptides can be endogenously processed to associate with Class-I and elicit CTL responses;
- the lipopeptide was safe and highly immunogenic in chimpanzees, whose immune system is similar to the human system;
- B and Th responses were induced in 6 out of 6 immunized chimpanzees and CTL were detected in 5 of 6 chimpanzees with diverse MHC background.

Further studies were performed with 5 additional palmitoyl-lysine peptides derived from the SALSA, the LSA1, the LSA3, the STARP antigens in mice, in Aotus and in Chimpanzees.

Contract number TS3*CT940345

The results obtained support the lipopeptide based approach since they showed that a very simple immunogen formulation, which can be used for vaccination in humans, is safe and strongly immunogenic: in chimpanzees strong T-lymphocyte proliferative responses were recorded to each of the five immunogens. Interferon gamma secretion, a cytokine known to have the most potent effect upon liver stage development, occurred in response to 4 out of 5 peptides. High to very high antibody titers were recorded to 4 of the immunizing antigens (1/5 does not define a B cell epitope, as shown by epidemiological studies). Moreover, the responses induced by lipopeptides injected in saline without adjuvant proved to be long lasting since they could be detected up to ten months after immunization. The relevance of the results obtained was demonstrated by specific B and T cell responses in the lipopeptide immunized animals towards native proteins on the sporozoite surface of *P. falciparum.* Similar results were also obtained in *Aotus* monkeys.

Finally, in four out of six cases lipopeptides injected in saline proved able to induce a HLA class I restricted, CD8 dependent, cytolytic activity. However, the specific activity obtained in this was in general terms relatively low (in the range 12 to 25% specific lysis), as is the case in people exposed to malaria in the field.

Altogether these results suggest that relatively large synthetic peptides, carefully chosen from critical areas of large proteins, and incorporating a simple palmitoyl-lysine which are simple to produce and purify under GMP conditions can induce, without adjuvant, a wide range of strong and long lasting immune responses in genetically diverse populations.

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Contract number ERBTS3*CT940346 (See ERBTS3*CT920053)

MALARIA PRE-ERYTHROCYTIC STAGES (MPES) EUROPEAN NETWORK ANTIGENS TARGET OF IMMUNE RESPONSES CAPABLE OF INHIBITING *P. FALCIPARUM* PRE-ERYTHROCYTIC DEVELOPMENT

Period: January 1, 1995 - December 31, 1997

Co-ordinator: INSTITUT PASTEUR, DEPT. PARASITOLOGIE BIOMEDICALE
Paris, France (P. DRUILHE)

Objectives

- Development of the immunology of MPES with the aim of developing an effective MPES vaccine.
- Acquisition of an improved knowledge of the biology of MPES.
- Improved coordination and exchanges within and between European and Developing Country teams.

Activities

The European malaria pre-erythrocytic stages network is aimed at elucidating the basic biology of, as well as developing control means against, human malaria. It includes:

* Molecular biology studies of pre-erythrocytic antigens from mostly *P. falciparum* and *P. berghei*, and in part *P. reichenowi* (identification, characterisation, production of genes and antigens: LSA1, a major 200 kDa molecule expressed in liver stages; SALSA, a 70 kDa antigen shared between sporozoite and liver stages; LSA3-729, a pre-erythrocytic stage specific molecule expressed in sporozoites and liver stages; DG21, a sporozoite specific 78 kDa molecule). Antigenic features of these molecules, conservation of epitopes amongst isolates, epitope mapping; immunogenicity in animals, characterisation and prevalence of immune responses in humans and in animals (mice and primates), identification amongst the remaining series of cloned pre-erythrocytic stage molecules of those that deserve further detailed studies.

* Improvement of the reproducibility of liver infections in Aotus monkeys. Immunisation, and sporozoites challenges, of chimpanzees and Aotus with *P. falciparum* antigens, of mice and Thammomys with *P. berghei*, *P. yoelii* antigens. Analysis of the immune responses developed by immunised animals and of the type of defence mechanisms operating. Comparison of the type of immunity induced by antigens and by whole parasites (i.e. irradiated sporozoites) in natural versus artificial hosts.

* Analysis of naturally occurring immunity to MPES in field conditions, of the mechanisms regulating parasite loads at the MPES level, and of the main antigens inducing such mechanisms. Analysis of the artificial immunity induced by injection of γ-irradiated sporozoites, and of the mechanisms and antigens responsible for such an immunity.

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* Studies of the mode of action and the respective importance of antibodies, antibody-cell cooperation, lymphocyte cytotoxicity and cytokines using *P. falciparum* and human hepatocytes, under *in vitro* conditions or *in vivo* in SCID mice.

Expected outcome

Our main goal is the development of an effective means of vaccination.

Additionally the project could provide:

⇒ An improved understanding of the human *P. falciparum* relationship at MPES level, mainly through an analysis of existing regulatory mechanisms developed against those stages by exposed individuals and their epidemiological consequences in various areas differing in their vectorial capacity.

⇒ Improved cohesion of European research in epidemiology and diagnostics in concertation with Developing Countries enabling a sustainable research strengthening in malaria endemic areas.

Results (so far)

In general, the malaria pre-erythrocytic stage network has provided a tremendous increase in the output of studies on the molecular biology, immunology and cell biology of human malaria liver stages.

Molecular biology

4 out of 4 of the new *P. falciparum* molecules being studied namely STARP, SALSA, LSA-1, LSA-3 have been characterized in terms of their full length DNA sequence and stage specific expression, and we have initiated the study of three new MPES genes, DG 64, DG 6F and DG 571, two of them in collaboration with Nijmegen and BPRC. Immunological studies with isolates of *P. falciparum* at sporozoite stage have shown the consistent expression of those genes and comparison of sequence data for areas of immunological interest of LSA-1 and LSA-3, have shown a remarkable degree of conservation, in contrast to many other *P. falciparum* antigens. Homologs of those genes have been found in the ape parasite *P. reichnowi* and for some of them the sequence (e.g. STARP) determined. Immunological and genetic screening of other plasmodial species led to identify an equivalent of LSA-3 in the rodent species *P. yoelii* which in particular share B- and T-cell epitopes with the *P. falciparum* gene and open the possibility to perform immunization and challenges with the rodent species in laboratory animals.

For immunization purposes, the genes have been cloned in various types of vectors, expressed in prokaryotic, eukaryotic cells and naked DNA vectors. For instance, a very large range of recombinant expression systems are now available for LSA-3 as a model system. This, together with the large number of lipopeptides derived from the above molecules, provides a very large range of immunization systems.

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Experimental models

One of the strengths of the network lies in the wide range of model systems available:

The primates facilities are unique, particularly in BPRC-TNO, in Cali (Columbia) and in CIRMF (Gabon) by their having chimpanzees and Aotus colonies, the connected excellent research laboratory facilities and the malarial expertise of the corresponding teams. Chimpanzees at BPRC have been fully MHC-Class-I and Class-II typed. In addition, steps have been taken to develop a novel model in Burma, the gibbon, as an experimental system for erythrocytic and liver stages.

The IMTPL in Belgium has developed a colony of Thamnomys which provided means to compare host parasite interactions in natural versus artificial hosts.

The unique set up in Nijmegen has provided a continuous production of large numbers of *P. falciparum* sporozoites for immunological studies and for challenges. In addition 2 insectaries were developed in Columbia for *P. falciparum* and *P. vivax,* one in IMTPL for *P. berghei* and another one in Paris for *P. yoelii.*

Despite the enormous difficulties of this model, the liver stage cultures of *P. falciparum* have been performed on a discontinuous but regular basis in Paris and used to assess *in vitro* defence mechanisms towards *P. falciparum* sporozoite and liver stages.

Many efforts have been devoted to develop a SCID mouse model enabling support *in vivo* for the *P. falciparum* liver stage development in human hepatocytes. Satisfactory progress has been made in this direction since the grafting of functionally active human hepatocytes has been successfully performed and kept for up to four months in this experimental host. However, attempts to infect those hepatocytes *in vitro* have up to now been unsuccessful.

Field set ups

Excellent field facilities have been developed and used for antigenicity studies mostly in Senegal, in Madagascar, but also in the Congo, in Kenya, in Brazil, in Burma and in the Gambia. The contribution of those centres has been invaluable for the identification of epitopes for B-cell, for T-helper cells and for cytolytic T-cells.

In addition, a novel type of field study has been designed and implemented to investigate the existence of naturally occurring anti-IMPES immunity. Some individuals appear to develop a strong resistance to sporozoite challenge and the correspondence between this and the immune responses developed to the molecules under study is expected to provide clues about which molecules induce responses which contribute to protection against MPES.

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Antigenicity studies

Using peptides and recombinants from the four lead molecules, studies in five endemic areas have identified the sequences defining B-cell epitopes and have showed high to very high sero-prevalences to them (e.g. 97% prevalence to LSA-3 repeats in Senegal spanning all age groups). Similarly T-helper cell epitopes were mapped and a correspondence between the level of malaria transmission and the proportion of T-cell responders was observed (up to 85% T-cell responders to most peptides in the Congo). Many of those epitopes were also found to be able to stimulate the secretion of interferon gamma, the cytokine known to be the most potent in blocking liver stage development.

One LSA-3 peptide induces the secretion of particularly high concentrations of this cytokine. Finally, a very large number of HLA class-I restricted CTL epitopes have been identified in the four genes under study: 6 in LSA-1, 2 in SALSA, 1 in STARP and 11 in LSA-3.

Immunogenicity and vaccination studies

Chimpanzees and Thamnomys were used as model systems to analyze the protection induced per irradiated sporozoites and the immune responses in the 18 krad chimpanzee paralleled those recorded in human volunteers immunized in the same manner.

However, the CD8 lymphocyte depletion planned in this animal could not be performed.

Rodent modelling revealed an inverse relationship between the susceptibility of the host to the parasite and the dose of irradiated sporosoites needed to achieve protection, i.e. protection was very difficult to induce in Thamnomys which is more susceptible than artificial laboratory hosts.

Using the four lead molecules, preliminary immunization attempts were performed in mice of five different H2 haplotypes with many different synthetic peptides and lipopeptides as well as recombinant proteins and a large range of adjuvants. One of the most remarkable results was obtained with lipopeptide which proved able to induce without, Th and CTL responses without adjuvant and to increase the immunogenicity to the point of apparently overcoming partially or totally the genetic restriction observed in bred mice. Therefore, immunizations were performed in chimpanzee using six lipopeptides injected in PBS and six non lipopeptides injected in montanide adjuvant, or adsorbed on microspheres.

By this means, T-helper cell responses were induced to all twelve peptides, some of them at very high level (stimulation indices > 80), antibody production was observed towards eleven of the twelve peptides, most of them at very high levels.

Moreover, these responses proved to be long lasting and to be specific to parasite native proteins. Finally, CTL responses were detected towards six of those peptides. Challenge studies indicated that SALSA and mostly LSA-3 held the best promise in terms of protection. A large number of immunization schemes were used in mice with LSA-3 as a model system to assess protection against *P. yoelii* challenge.

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Results with QS21, titermax, FCA or FIA were disappointing. To date the best results were obtained with lipopeptide or with microsphere immunization which have the advantage of being effective without adjuvant and to induce a full range of immune responses.

In vitro study of defence mechanisms

The *P. falciparum* human hepatocyte *in vitro* system was used to study defence mechanisms. At very low concentration, antibodies to STARP and LSA-3, and also anti-SALSA antibodies strongly inhibited *P. falciparum* sporozoite invasion. IgM from irradiated sporozoite volunteers proved more efficient than IgG antibodies.

Several attempts to show whether CTL cells could lyse human or chimpanzee infected hepatocytes have been made so far without reaching a conclusive result. However the conditions necessary to achieve this goal have considerably improved. For instance, a method enabling the raising of CTL malaria specific CTL lines from healthy volunteers, developed in Oxford, increases greatly the chances of matching the MHC Class-I antigen from the effector cells with that of the target hepatocytes. One drug, already developed and used clinically, proved to be very effective *in vitro* upon *P. falciparum* liver stages but not against *P. yoelii* liver stages. G-Oligonucleotide primers derived from LSA-3 were used in a PCR assay which proved to be the most sensitive diagnostics mean available to date to detect very low grade *P. falciparum* blood infection.

In total, these studies have confirmed the potential of the molecules under study, which antigenicity and immunogenicity as well as conservation among isolates appeared to be remarkable.

The vaccination schemes employed up to now may not yet be optimal, but have yielded very .encouraging results, and the conditions to assay the efficacy of further immunization schemes have greatly improved.

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Contract number IC18*CT950015

THE AFRICAN MALARIA VACCINE TESTING NETWORK

Period: January 1, 1996 - June 30, 1998

Co-ordinator: NATIONAL INSTITUTE FOR MEDICAL RESEARCH,
Dar es Salaam, Tanzania (W. L. KILAMA)

Objectives

To establish an African malaria vaccine testing network. To facilitate coordinated multicentre malaria vaccine studies.

Activities

* Establish a secretariat for the network at the National Institute for Medical Research (NIMR) in Dar es Salaam.

* Convene meetings of the network coordinating committees.

* Familiarize the coordinating committee with potential field study sites.

* Distribute questionnaires, collate questionnaires and publish a database and a booklet on African institutions undertaking malaria vaccine research and on potential field sites for malaria vaccine trials.

* Suggest programmes for training of African scientists in various aspects of malaria vaccine trials, such as good clinical practices, good laboratory practices, data management.

Expected outcome

Publishing of a database and a booklet on African institutions undertaking malaria vaccine research and on potential field sites for malaria vaccine trials. Evaluation of potential field sites for malaria vaccine trials. Initiation of courses relevant to malaria vaccine trials. Creation of a forum for exchange of experiences regarding operational/logistical difficulties, as well as for scientific discussions.

Contract number IC18*CT950015

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Contract number IC18*CT950016

SELECTION OF *P. FALCIPARUM* GENES FOR MPES VACCINE DEVELOPMENT

Period: January 1, 1996 — December 31, 1998

Co-ordinator: INSTITUTE PASTEUR, BIO MEDICAL PARASITOLOGY, Paris, France (P. DRUILHE)

Objectives

- To further investigate the vaccine potential of SALSA, STARP, LSA1 and LSA3.
- To improve our understanding of the mechanisms mediating protection against *P. falciparum* MPES in humans.
- To investigate the potential of 10 novel MPES genes and to choose those which deserve further characterization and immunogenicity studies.

Activities

* Using the 4 lead antigens (Ags) SALSA, LSA1, STARP and LSA3, whose antigenicity is established, we will employ a series of antigen-presentation systems, namely lipopeptides, multiple antigen lipopep-tides, recombinant Ags adsorbed to microparticles, DNA-based immunization, in order to optimize the conditions under which defence mechanisms are induced in chimps, in Aotus against *P. falciparum* and in mice against *P. yoelii*. Where necessary improved recombinant expression systems will also be evaluated.
* Perform analysis of B, Th, CTL responses, and identify antigens targeted by mechanisms effective under *in vitro* conditions, so as to provide an understanding of the basis of protection and therefore develop adapted formulations.
* Develop improved *in vivo* and *in vitro* screening systems to evaluate the 4 lead Ags. Compare antigen specific immune responses in humans and in chimpanzees immunized with irradiated sporozoites. Analyze the relationship between immune responses and protective status in individuals exposed in endemic areas. Analyze *in vitro* with the human and chimpanzee hepatocyte model the various effector mechanisms directed to these antigens. Immunize and challenge chimpanzees, Aotus and mice. Additional studies in rodents, in immunocompromised animals reconstituted with human hepatocytes, and in exposed populations, are aimed at supplying an understanding of the immunological basis of protection in natural host-parasite combinations (*P. berghei* in Thamnomys, *P. falciparum* in humans and human hepatocytes) and particularly studying CTL activity.
* Employ the conditions of immunization defined in the above steps to investigate the potential of a further 10 novel antigens.
* Extend the "best" antigens and presentation systems, identified in these studies, mostly in chimpanzees and Aotus, to a larger groups of Aotus so as to gather statistically significant data.

Expected outcome

A strengthened network of EC and DC partners, with a better understanding of the critical effector mechanisms that protect humans against *P. falciparum* pre-erythrocytic stage parasites. From this improved understanding we expect to develop a rational strategy for human MPES vaccine development.

Contract number IC18*CT950016

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Contract number IC18*CT950019

EVALUATION AND DELIVERY OF THROMBOSPONDIN RELATED ANONYMOUS PROTEIN (TRAP) IMMUNOGENS FOR THE DEVELOPMENT OF MALARIA VACCINE

Period: January 1, 1996 - December 31, 1998

Co-ordinator: IMPERIAL COLLEGE OF SCIENCE TECHNOLOGY AND MEDICINE, INFECTION AND IMMUNITY SECTION, London, United Kingdom (A. CRISANTI)

Objectives

To evaluate, in different models, the ability of TRAP derived immunogens to elicit protective immunity against a challenge with Plasmodium sporozoites.

Activities

* Selection of delivery systems for inducing humoral immunity and specific CTLs against TRAP. Few antigen delivery systems, have been shown to induce both humoral immunity and specific CTLs, these include: recombinant vaccinia viruses; lipidic adjuvants; naked DNA; listeriolysin O (LLO) of *L. monocytogenes*. We will compare the relative efficacy of these delivery systems to induce an appropriate immune response against *P. berghei* TRAP immunogens containing B cell, Helper and CTL epitopes. Cell lines will stably transfected with a series of DNA vectors inducing the expression of progressively deleted PbTRAP constructs.

* Analysis of TRAP epitopes variability among *P. falciparum* isolates. Polymorphisms of immunologically relevant epitopes will render problematic the use of TRAP for the development of a vaccine. A total of one hundred blood samples will be collected from different locations of endemic areas in Mali at different time points during the malaria transmission season. Parasite DNA will be extracted from bloodspots and amplified with combinations of primers generating sequences of immunologically relevant T and B cell epitopes permitting direct DNA sequencing to look for mutations.

* Cloning of TRAP homologues from *P. knowlesi*, *P. cynomolgi* and *P. fragile*. There are no perfect host systems that can be used to model *P. falciparum* infection in humans. Aotus and Saimiri genera are variably susceptible to blood stage infections from a limited range of adapted parasite species, a challenge model with Aotus is limited to the use of particular monkey subspecies (*Aotus lemurinus*) and to a particular parasite strain (*P. falciparum* St. Lucia). Specific PCR products will be generated from parasite DNA using primers for regions of TRAP that are conserved between the Plasmodium species. Labelled PCR products will be used as probes to screen λgt 10/λgt 11 genomic libraries developed from DNA of *P. knowlesi*, *P. cynomolgi* and *P. fragile*.

* Evaluation of experimental induced PfTRAP immunity against Plasmodium sporozoite challenge. The TRAP gene from the St. Lucia strain will be amplified in PCR

experiments, cloned and sequenced. A cohort consisting of 10 adult animals of either sex will be randomly assigned to three experimental groups of five animals each. The two groups of monkeys will be immunized with constructs generated using either St. Lucia TRAP or a polymorphic PfTRAP variant. After thorough analysis of humoral and cellular responses all the monkey cohorts will be challenged with 20,000 St. Lucia sporozoites. Monkeys will be observed daily for parasitemia.

Expected outcome

It is anticipated that these experiments will allow us to select an appropriate delivery system for TRAP derived immunogens inducing both humoral and cell mediated cytotoxic immunity. This information will provide the rationale for developing an experimental TRAP vaccine for human trials.

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Contract number IC18*CT950020

A CONCERTED EUROPEAN APPROACH TOWARDS THE DEVELOPMENT OF MALARIA VACCINES

Period: January 1, 1996 - June 30, 1998

Co-ordinator: STATENS SERUM INSTITUT, OFFICE OF INT. HEALTH, Copenhagen, Denmark (S. JEPSEN)

Objectives

To promote a coherent approach to the development of malaria vaccines. This requires focused interactions between vaccine industrialists and scientists in Europe and developing countries, concerned with malaria antigens and with the wider fields which underpin malaria vaccine development. To identify and exploit existing structures and resources to support malaria vaccine develop-ment and to create fora for the regular exchange of information on planned work, progress and results germane to malaria vaccines. To provide a channel for expert advice on malaria vaccine research and development to the European Commission, as well as to other national and international authorities. To develop partnerships amongst academia, the Public Sector Vaccine Institutes and the European Vaccine Enterprises and to promote interaction amongst those engaged in malaria vaccine research and development in Europe and elsewhere.

Activities

* The primary core activity of this concerted action will be a series of expert meetings addressing different aspects of malaria vaccine development. These meetings will bring together groups of the INCO-DC (and former STD3) contract holders, other experts in the fields which underpin the science base of a malaria vaccine, and representatives of vaccine manufacturers. The industries and public sector institutes invited to specific meetings include: SmithKline Beecham (Belgium), Pasteur Merieux (France), Chi-ron/ BIOCINE (Italy), Hoffman LaRoche (Switzerland), Swiss Vaccine and Serum Institute (Switzerland), Statens Seruminstitut (Denmark) and RIVM (The Netherlands). Liaison with the European Vaccine Manufactures (EVM) is ensured.
* Assistance tools will be updated and made operational: The Malaria Antigen Database (with WHO/TDR, USAID and NIAID), The compendium of in vitro and smaller animal models (with COST/STD and EVM), the PVEN document (with PVEN) and the "Atlas" and guidelines for field trials (with AMVTN).

Expected outcome

Vaccines are the most cost-effective approach to control of transmissible diseases. The benefit of this CA is in its role in expediting and rationalising progress towards malaria vaccine development and production. Continual discussions with representatives from the malaria endemic countries will benefit the process of malaria vaccine development. Developing countries will benefit from the proposed action, and also from the networks (PVEN and AMVTN) which will enhance the influence of the Developing Country partners on the process of vaccine development.

Contract number IC18*CT950020

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PRE-CLINICAL STUDY OF THE IMMUNOGENICITY OF MPS3 AND GLURP TWO *P. FALCIPARUM* ANTIGENS TARGETED BY PROTECTIVE ANTIBODIES

Period: January 1, 1996 - December 31, 1998

Co-ordinator: STATES SERUMINSTITUT, KLINISK BIOKEMISK AFDELNING, Copenhagen, Denmark (S. JEPSEN)

Objectives

To optimize the immunogenicity of MSP3 and GLURP by using several antigen-presentation systems in a) mice, b) Saimiri, c) Aotus, and d) Chimpanzee. To characterize the antibodies (fine epitope specificity, isotype) induced by the various protocols, and analyze their biological effect in defence mechanisms, a) in vitro, invasion and ADCI, b) *in vivo* by passive transfer in the humanized SCID mouse and in c) primate. Compare the results with *P. falciparum* challenge experiments in the primates immunized with MSP3 and GLURP. Complete the characterization of the B and T cell epitopes from MSP3 and GLURP by epidemiological field studies.

Activities

* Immunizing mice with one lipopeptide, and one recombinant from MSP3 and one peptide and one recombinant derived from GLURP with the different adjuvants. The titer, and isotype as well as the ADCI effect of the antibodies obtained will be determined.

* Aouts will be BCG primed, immunized with PPD-coupled GLURP and MSP3 and subsequently challenged with *P. falciparum.*

* ADCI experiments will be conducted in SCID mice harbouring live *P. falcuparum* and human monocytes by passively transferring total IgG from hyperimmune individuals to confirm the model. Anti-R0, anti-R2, and anti-MSP3 and other antibodies will subsequently be analyzed in the SCID mode.

* Epidemiological studies with MSP3b and R0, and R2 in Dielmo.

* Mice will be immunized with new peptides and recombinant proteins derived from MSP3 and GLURP with the best performing adjuvant. The titer, and isotype and ADCI effect of the antibodies induced by new constructs will be determined.

* Immunizing Aotus and Saimiri with the GLURP and MSP3 vaccine formulation that induced antibodies which prove to be efficient in ADCI. The humoral and cellular immune responses of the monkeys will be analyzed.

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* The antibody reactivity to epitopes identified in MSP3 clone 256 and 256B and peptides derived from GLURP will be analyzed in the population of Dielmo.

* Immunizing Aotus and Saimiri with antigens provided that they are superior to the initial constructs. The immunogenicity of the best performing MSP3 and GLURP antigen formulation will be determined in Chimpanzees.

Expected outcome

The optimal conditions for inducing antibodies against MSP3 and GLURP will be determined and functional protection assays established. Improved understand of the critical epitopes involved in the production of protective antibodies and cross-reactivity to MSP3 and GLURP.

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Contract number IC18*CT950022

THE APPLICATION OF TRANSFECTION TECHNOLOGY TO MALARIA VACCINE DEVELOPMENT

Period: January 1, 1996 - December 31, 1998

Co-ordinator: UNIVERSITY OF LEIDEN (RUL), DEPT. OF PARASITOLOGY, Leiden, The Netherlands (A.P. WATERS)

Objectives

To develop DNA expression vector systems that will facilitate the introduction of genes encoding proteins with vaccine potential into asexual bloodstages of the rodent malaria parasite, *Plasmodium berghei*. To study the following model genes: Apical membrane antigen (AMA) - 1, erythrocytic vaccine candidate Pbs21 ookinete surface protein, transmission blocking candidate Circumsporozoite (CS) protein, pre-erythrocytic vaccine candidate. To clone and modify these genes in such a way that they can be expressed in recombinant *P. berghei* parasites with a view to analyzing the regulation of their expression and manipulating the immune response to these proteins. To isolate DNA elements (promoters) involved in the control of the stage specific expression of the three P. berghei genes to permit the appropriate expression of recombinant genes upon re-introduction into the parasite. To attempt to knock out these genes in the *P. berghei* parasite genome and demonstrate their immediate biological function and essential nature. To re-introduce modified copies or analogues of the genes into the knock-out mutants to 1) restore 2) modulate 3) demonstrate the conserved nature of the function of the encoded protein.

Activities

* Appropriate vectors for the expression of genes introduced by genetic transformation into the rodent malaria, *P. berghei*, will be developed based upon the available DHFR/TS selectable marker which donates resistance to the antimalarial drug, pyrimethamine.
* Attempts will be made to develop new selectable markers and vectors for the disruption and modulation of genes.
* The relatively new system for the transfection of malaria parasites will be disseminated throughout the partner groups.
* The technology for the dissection of promoter structure will be developed and disseminated.

Expected outcome

The study should provide a case example of the utility of transfection technology, as applied to malaria parasites, to investigate the function as well as the immunological and biochemical properties of conserved proteins of malaria parasites that are considered to be candidate components of vaccine formulations. An insight will be gained into the functional structure of stage specific promoters of gene transcription. This can be expected to include an identification of those elements which dictate stage and sex specificity and those which direct basal transcription.

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Contract number IC18*CT960125

CONCERTED ACTION IN SUPPORT OF HIGH QUALITY NON-HUMAN PRIMATE (NHP) BREEDING AND BIOMEDICAL RESEARCH IN NHP SOURCE COUNTRIES

Period: September 1, 1996 - August 31, 1999

Co-ordinator: BIOMEDICAL PRIMATE RESEARCH CENTRE,
DEPT. OF PARASITOLOGY, Rijswijk, The Netherlands
(A. W. THOMAS)

Objectives

- To develop an organisational and communication framework between European and Developing Country primate research centres within which improvements in the capabilities for research on health problems of Developing Countries and improvements in animal welfare can most effectively be realised.
- To identify the areas for improvement that can most benefit from collaborative efforts and co-ordinate the implementation of such efforts.

Activities

* Estab lish Regional Networks and appropriate regional centres for Latin America, Africa and Asia. Each regional centre will inform primate centres within the region of the establishment of PVEN and invite applications to participate in the network. Each regional centre will, together with the PVEN advisory board, select 5 - 6 appropriate members who will form the initial core membership of PVEN. A European co-ordinating centre will be established and the PVEN regional centres, together with this European centre, will invite independent experts to join an external advisory board for the network.
* Hold regional meetings at which the opportunities for collaborative development of improved Research and Reference standards will be identified. Specifically the Reference Programme (published in PVEN Guidelines) will be used as a starting point for the evaluation of critical factors in:
  - — colony health and management including virological, bacteriological, fungal and parasitological testing, the implementation of welfare standards, haematology and clinical chemistry (normal and pathological values).
  - — colony characterisation and development including MHC typing, DNA fingerprinting, genetic conservation of laboratory species, in and out-breeding, reproduction technology.
  - — research fundamentals including cytokines, hormones, blood groups, MHC, CD markers, cellular immunology techniques, non-invasive monitoring.
  - — knowledge resources including computer databases.
  - — training including exchange of staff to transfer specific technologies and the establishment of accepted standards of training in primate care and use for animal handlers.
* Identify the most appropriate sources for expertise and ensure such expertise is available to primate centres, through support of travel, organisation of workshops, etc. in order to assist in the implementation of collaborative efforts.

Contract number IC18*CT960125

Expected outcome

⇒ Clearly identified priorities for collaborative development of research and reference capabilities.

⇒ Management and communication structures that allow rapid and free exchange of information between primate centres.

⇒ Co-ordination of the improvement of capabilities, ensuring that duplication of effort is substantially reduced and that optimal use of scarce resources is achieved.

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Contract number IC18*CT970238

IMMUNITY TO GENETICALLY CHARACTERISED MALARIA INFECTIONS: A COMPARISON BETWEEN LONGITUDINAL STUDIES IN A WEST AND AN EAST AFRICAN VILLAGE EXPERIENCING DIFFERENT INTENSITIES OF TRANSMISSION

Period: October 1, 1997 - September 30, 2000

Co-ordinator: UNIVERSITY OF EDINBURGH, INSTITUTE OF CELL ANIMAL AND POPULATION BIOLOGY,
Edinburgh, United Kingdom (D.E. ARNOT)

Objectives

To compare the molecular parasitology and immunology of antigenic variation in *P. falciparum* malaria in an area of high transmission and hyperendemic malaria (Ghana, West Africa) with that in an area of hypoendemic, unstable malaria (Sudan, East Africa) and to discover whether parasite strategies for immune evasion by antigenic variation vary under different transmission conditions.

Activities

* Cloning and sequence PfEMP-1/*var* genes and gene fragments from African *P. falciparum* samples to obtain reagents and develop assays for the analysis of the genetics of the PfEMP-1/*var* system and to test whether clonal antigenic variation associated with *var* gene switching allows the parasite to survive in the host during chronic malaria infections.

* Characterising the immunological and parasitological events associated with the acquisition of agglutinating antibody responses. To attempt to correlate agglutination responses in human infections from Sudan and Ghana with the appearance of specific responses to defined parasite encoded adhesion molecules such as PfEMP-1 and sequestrin. Building on ongoing research, to compare the acquisition of agglutinating antibody responses with the acquisition of antibody responses against variant and conserved portions of major parasite merozoite antigens such as MSP-1.

* Strengthening molecular biology and immunology research on malaria in the African partner institutions and to train junior scientists in the European partner laboratories. To promote scientific exchanges between malaria researchers in East and West Africa.

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Expected outcome

By the end of the study we will have basic epidemiological data on the period that individuals remain asymptomatically infected at each study site and quantitative data on whether parasite densities fluctuate or remain broadly stable during asymptomatic infections at each site. The length and densities of gametocytaemia will also be known. This epidemiological data will allow us to interpret the results of the concurrent immunological and molecular biological studies on typing the PfEMP-1/*var* genes (using RT-PCR and human phage display library generated antibodies) in the parasites isolated from symptomatic and asymptomatic individuals.

This will allow us to assay if, and how frequently, the parasite population in the monitored asymptomatic infections is switching PfEMP-1 expression and whether asymptomatic individuals infected with an apparently genetically stable population of parasites show constant responses in mixed agglutination assays or whether these vary in concert with *var* gene switches.

Such reagents and information will be essential for further studies on the role of *P. falciparum* antigenic variation in pathogenesis.

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MATHEMATICAL MODELS OF THE IMMUNOLOGICAL AND CLINICAL EPIDEMIOLOGY OF PLASMODIUM FALCIPARUM MALARIA

Period: September 1, 1997 - August 31, 2000

Co-ordinator: EBERHARD-KARLS-UNIVERSITY, DEPT. OF MEDICAL BIOMETRY, Tübingen, Germany (K. DIETZ)

Objectives

- Construct mathematical models of the immunological and clinical epidemiology of *Plasmodium falciparum (P.f.)* malaria, and test the model predictions against observations collected independently of this project.
- Use the models for helping to:
  a) calculate the expected impact of interventions;
  b) improve the design of field intervention trials;
  c) identify crucial gaps in knowledge that constrain (a) and (b).

Activities

* Review of relevant biological, clinical, and epidemiological knowledge
* Specification of observations to be used as modelling targets:
  a) longitudinal data on the behaviour of *P.f.* infection and disease in individuals after one or more experimental inoculations;
  b) longitudinal data on the behaviour of *P.f.* infection and disease in individuals naturally exposed to repeated inoculation;
  c) longitudinal data on the transmission of *P.f.* and the behaviour of *P.f.* infection and disease in naturally exposed populations.
* Formulation (and reformulation) and programming of the models.
* Design, performance and evaluation of simulations.
  The evaluation will consist primarily of the formal comparison of simulations to observations. The comparisons will suggest revisions of the models' assumptions and parameters values, until an acceptable fit is obtained.
* Formulation of recommendations
  The practical implications of the work will be made explicit, leading to recommendations in the following areas: (a) planning of malaria control; (b) evaluation of malaria control programmes and of field trials; (c) epidemiological research; (d) research on statistical methods.

Expected outcome

Better simulation models will help to clarify the following issues:

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1) Optimal adaptation of control strategies, using currently available methods, to different epidemiological and socio-economic situations, including projections of what could be expected from additional resources;

2) Expected benefits from new tools likely to be technically feasible, including different kinds of vaccines;

3) Optimal development and evaluation strategy of such new tools.

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Contract number TS3*CT920045

PLASMODIUM VIVAX MALARIA IN MAN: ANTI-DISEASE AND TRANSMISSION BLOCKING IMMUNITY

Period: November 1, 1992 - October 31, 1995

Co-ordinator: UNIVERSITY OF EDINBURGH, INST. OF CELL, ANIMAL AND POPULATION BIOLOGY, Edinburgh, United Kingdom (R. CARTER)

Objectives

- To study the mechanisms of immunity to disease in *P. vivax* and *P. falciparum* malaria.
- To study immunity to transmission of *P. vivax* malaria in human populations.

Activities

* The disease characteristics of *P. vivax* and *P. falciparum* infections in naive (non-endemic) individuals and in individuals from an endemic population were recorded and characterised. Cellular and physiological responses were measured both *in vivo* and *in vitro* in response to malaria exo-antigens. The presence of antibodies in convalescent serum was assayed for ability to block the induction of cytokines and other factors by the action of malarial exo-antigens on human blood monocytes *in vitro*.
* The phenomena of paroxysm in *P. vivax* malaria was the subject of special investigations to define the cellular mechanisms and soluble mediators involved. Attempts were made to characterise and fractionate the parasite products associated with the induction of a paroxysm including investigation of species-specific aspects of their function using products from blood stage parasites of the two human malaria parasite species found in Sri Lanka, *P. vivax* and *P. falciparum*.
* Target antigens of transmission-blocking immunity against *P. vivax* were studied and characterised by attempts to clone the genes coding for these proteins. Using a panel of monoclonal antibodies against *P. vivax* gamete surface antigens previously developed in our laboratories, we screened eukaryotic recombinant DNA expression libraries.

Results

Disease profiles of successive infections of *P. vivax* and/or *P. falciparum* were studied in an endemic population in the south of Sri Lanka. A total of 1.748 cases of diagnosed malaria were recorded in a population of 1.942 individuals over a period of 18 months. Analysis of these infections showed that there was a tendency for successive infections within a period of about six months to be of lower clinical severity. This was especially true of infections preceeded by a *P. vivax* infection; prior infection with *P. falciparum* gave very little protection against *P. vivax* infections and only slight protection against homologous *P. falciparum*.

Ex vivo studies were carried out on the changing properties of cells and soluble mediators before, during and after a paroxysm of *P. vivax* malaria. Using cytokine-specific antibodies

to neutralise their effects on cells *in vitro*, we have now shown that the cytokines TNF, GM-CSF and IL-2 are all critically involved in events taking place during a *P. vivax* paroxysm. By the same approach the cytokines IL-la and IL-lb, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IFNγ and TGFβ have no detected activity during such a paroxysm. The active cytokines, TNF, GM-CSF and IL-2, and the active parasite products released just before a paroxysm have been shown to mediate their effects through the presence of monocytes; a role for T cells is implicated by the active involvement of IL-2, but direct evidence for the involvement of T cells has not yet been obtained.

In our cell assay, which mediates inactivation of the sexual blood stage parasites of *P. vivax*, the active parasites present in plasma during a paroxysm have been shown to be antigenically species-specific.

A synthetic peptide was made representing a sequence of *P. vivax* antigen present on both sexual and asexual blood stage parasites. This peptide is recognised by a monoclonal antibody, A12, which is capable of totally blocking the infectivity of *P. vivax* gametocytes to mosquitoes. Antibodies raised against this peptide also induced *P. vivax* transmission blocking antibodies. A gene has been cloned and sequenced whose product contains the peptide sequence recognised by the A12 Mab; it is not known, however, if this gene encodes the protein which is the target of the transmission blocking activity of Mab A12.

A second gene has been cloned from a COS cell expression library of *P. vivax* by panning with Mabs with transmission blocking activity against *P. vivax*. The product of this gene is the target of a Mab, G13, whose epitope specificity is distinct from that of A12.

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Contract number TS3*CT920063

THE BIOLOGICAL AND EPIDEMIOLOGICAL SIGNIFICANCE OF TRANSMISSION BLOCKING IMMUNITY AND TRANSMISSION REDUCING FACTORS IN ENDEMIC FALCIPARUM MALARIA. HUMAN FACTORS THAT INFLUENCE INFECTION OF MOSQUITOES

Period: January 1, 1993 - December 31, 1995

Co-ordinator: KATHOLIEKE UNIVERSITEIT NIJMEGEN, DEPT. OF MEDICAL PARASITOLOGY, Nijmegen, The Netherlands (J.P. VERHAVE)

Objectives

- To study epidemiologically and experimentally the transmission potential of blood from gametocyte carriers.
- To identify human blood factors that can reduce infection of mosquitoes.

Activities

* Infectivity of gametocytes will be studied in the Yaoundé region by selecting gametocyte carriers and studying naturally and experimentally infected mosquitoes. The blood will be examined locally for gametocyte density, *in vitro* fertilisation in original and normal plasma-replaced blood and gametocyte antibodies; contents of mosquito stomachs will be examined for phagocytosis, percentages of zygotes and oocysts and their quantities.
* Mosquitoes fed on whole patient blood provide an indicator for the overall transmission capacity of the individual gametocyte carrier. The difference between the mean number of oocysts in the control batch of mosquitoes and the experimental batch provides information about the presence of transmission blocking factors in the plasma sample.
* The percentage of white blood cells containing parasite material is determined in the dissected bloodmeal, 3 hours after ingesting, using the monoclonal antibody against 25kD protein, conjugated with FITC. Numbers of fertilised macrogametes/ookinetes are counted in bloodmeals after 12-24 hours, using the FITC-labelled the anti-25kD monoclonal antibody.
* Oocysts on the mosquito midguts are counted after one week, using an ordinary light microscope.
* Transmission-blocking activity will be studied in age-graded populations with varying degrees of transmission-intensity, and longitudinally over the season.
* The overall transmission blocking capacity can be split into the various stages of development within the mosquito and the reducing action of human factors can be studied in detail. Series of data will be built up on female gametocytes, gametes or zygotes, retort forms, ookinetes and oocysts.
* Sera collected from gametocyte carriers are to be tested for antibodies, particularly when their IgG shows blockage in the bioassay. The tests to be used are the anti-48/45 ELISA, and the anti-230 kD ELISA.

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Results

Achievements of transmission research up to October 1996

Analysis of factors determining the outcome of gametocyte-containing blood meals by (reared) *Anopheles gambiae* in Yaoundé, Cameroon, indicated that gametocyte density is related to mosquito-infection and to numbers of oocysts. Transmission was obtained in about 50-75% of the experimental feeds. Sex ratios did not correlate with mosquito-infection. Fever and parasitaemia did not influence success of mosquito-infection, but the sickle trait did.

Experimental infections were carried out with blood of some 350 gametocyte carriers, with the aim of identifying natural transmission-blocking (TMB) factors in the blood that determine the outcome of sporogonic development. A comparison was made between infections with blood containing autologous plasma and blood in which the plasma was replaced with that from a donor without previous malaria exposure or prophylaxis. Replacement resulted in a significant increase in mosquito-infection, especially when the homologous plasma was washed away. This indicates that plasma factor(s) can reduce the transmission capacity of gametocyte carriers to mosquitoes.

This reduction is now being compared with assays using cultured gametocytes of NF54 in Nijmegen and sera from the same gametocyte carriers. Inconsistencies in the comparisons, due to difference in procedures and in levels of infection in nature and in the laboratory are met by careful washing away of the carriers' plasma. The numbers of mosquitoes examined must be large enough for statistical analyses. The value of dissecting 50-100 mosquitoes in a batch process is being assessed. The mosquito strain used in Yaoundé appeared completely susceptible to NF54.

Antibodies of sexual stages

The various types of antibody tests for sexual stage antibodies are correlated with reduction of transmission. Confirmation has been obtained of the TMB action of an anti-Pfs25 mab, using the blood from gametocyte carriers; the percentage mosquito-infection was reduced 10 times. The mab, based on isolate NF54, acts against sporogonic stages of all isolates tested in Yaoundé carriers.

Mabs anti-Pfs48/45 were used to develop a competition ELISA (cELISA), to detect natural Pfs48/45 antibodies. Agreement between cELISA (5 epitopes) and TB activity in Cameroonian gametocyte carriers was about 75%. In healthy schoolchildren it was not significant. A specificity of 94% was found and a sensitivity of 44%: a positive test indicates TMB in the mosquito assay, but a negative result does not exclude TMB. Gametocytes generally express the lic epitope (PCR-confirmed).

Population studies in rural areas with different transmission intensity resulted in low prevalence of Pfs48/45 using the cELISA, whereas, some 66% of sera from Cameroonian gametocyte carriers was positive. European carriers all developed TMB and epitope-specific antibodies.

Also a cELISA demonstrating antibodies against Pfs230 has been developed. All sera from gametocyte carriers immunoprecipitated Pfs230 and blocked transmission by 50%. However, no correlation exists between these two phenomena. All anti-Pfs230 mabs of IgG2 isotypes blocked transmission only in the presence of complement, whereas IgG1 mabs did not. The hybridoma cell line producing non-blocking mab of the IgG1 isotype was switched to isotypes IgG2b and IgG2a. These switched mabs effectively blocked transmission by lysis of macrogametes and zygotes in the presence of complement. Using these techniques we have fulfilled our aim to identify the TMB activity of Pfs230 ab; the cELISA with Pfs230 mab did not reveal TMB activity in sera from gametocyte carriers.

Isolates from Yaounde-patients were cultured, along with isolate NF54, gametocytes have been produced, but infections were not successful.

Gametocytogenesis and gametogenesis

Patients with high asexual parasitaemia received amodiaquine or sulfadoxine-pyrimethamine medication and returned after one, and up to four weeks for detection of gametocyte density, infectivity and antibodies. Existing parasitaemias were gone, as well as the elevated levels of acute phase protein CRP. Fansidar treatment induced an increase of gametocyte densities after one week, whilst infection often failed. From 2 weeks all gametocytaemias decreased as did infection success. A similar wave of circulating gametocytes was found in rural Mengang, after treatment with chloroquine, but mainly in patients/carriers with parasites resistant to chloroquine.

Gametocyte-maturity in the circulation and phagocytosis in the bloodmeal are presently being studied in terms of transmission success. Removal of WBC increases mosquito infections.

Transmission in the field

Progress was made in the detection of sporogonic stages inside the mosquito, using the anti-Pfs25 mab-FITC, both in the field and in the lab. A major reduction was found to occur directly after the bloodmeal. In the transition from macrogametes/zygotes to ookinetes about 60% are lost, the other 40% forming ookinetes to young oocysts. Thereafter the loss is minimal. Research is now concentrated on major loss and the mechanisms involved.

Bloodfed mosquitoes were collected in houses in a rural village with hyperendemic malaria and parasites in gut-contents examined with immunofluorescence on the spot. 12% of *An. gambiae* or *An. funestus* mosquitoes contained zygotes or ookinetes over a period of 24 hours after a bloodmeal. Sporozoite rate was 5%. The gametocyte index was 38% with 8/µl (counted against 1000 WBC) and was maximal in houses with the highest number of infected mosquitoes. Gametocyte detection done with QBC, reveals about twice as many gametocyte carriers. Using fed mosquitoes caught under bednets with gametocyte carriers, again a significant correlation was found between gametocyte density, the percentage of infected mosquitoes and number of developing parasites in the mosquito midguts. Timing of developing zygotes, retort forms and ookinetes was registered. Only carriers who carried over 300 gametocytes/µl gave rise to young oocysts at about 40h after the bloodmeal.

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Contract number IC18*CT950018

THE DEVELOPMENT AND POTENTIAL OF MIDGUT ANTIGENS AS VACCINE AGENTS FOR NOVEL MOSQUITO AND MALARIA CONTROL

Period: January 1, 1996 - March 31, 1997

Co-ordinator: UNIVERSITY OF ABERDEEN, DEPT. OF ZOOLOGY, Aberdeen, United Kingdom (P.F. BILLINGSLEY)

Objectives

To establish that sera raised against mosquito midgut can interfere with normal mosquito biology. To demonstrate that anti-mosquito antibody at physiological titres can adversely affect vector longevity and fertility. To demonstrate that anti-mosquito antibody at physiological titres can reduce malaria transmission.

Activities

* New colonies of mosquitoes, *Anopheles gambiae ss* and *Anopheles tessellatus*, will be established at two sites. These and established colonies will be used as the sources of new antigen preparations from midgut-derived tissues. Screening of some 200-300 monoclonal antibodies (MABs) directed against *An. stephensi* tissues will be initiated early in the project, and continued throughout.

* Newly derived sera, previously characterized sera and MAbs will be screened by a standardized approach for anti-mosquito (fecundity and longevity) activity, and for their effects on transmission of human and malarial parasites. Sera and MAbs will be characterized by IFA and Western blot.

* Promising antigen preparations will be further refined for detailed study, including production of new MAbs. Mosquito cDNA libraries will be prepared and screened in preparation for production of recombinant antigen. Preliminary sequencing of identified antigen cDNA may be undertaken.

* Promising antigen preparations, and resulting sera and MABs will be subjected to intense screening at all sites, and to more detailed characterization at the molecular level. Studies will include colony and wild-caught mosquitoes.

Expected outcome

Promising antigen preparations, and resulting sera and MABs will be subjected to intense screening at all sites, and to more detailed characterization at the molecular level. Studies will include colony and wild-caught mosquitoes.

Contract number IC18*CT950018

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Contract number TS3*CT920084

THE DEVELOPMENT OF A PHARMACOLOGICAL MODEL FOR ANTI-MALARIALS WHICH INTERFERE WITH PHOSPHOLIPID METABOLISM

Period: January 1, 1993 - December 31, 1995

Co-ordinator: CNRS, URA 1856, INTERACTIONS MEMBRANAIRES (UNIV. MONTP. 1), Montpellier, France (H. VIAL)

Objectives

To study novel antimalarial agents that act upon phospholipid metabolism in the erythrocytic stages of malarial development, and to develop appropriate pharmacological models.

Activities

This project aims to find new chemotherapeutic treatments (and eventually prophylactics) for malaria. Interruptions of phospholipid metabolism of erythrocytic stages of *Plasmodium*, which is essential for the synthesis of parasite membranes, blocks parasite development. The most promising compound inhibits choline carrier, a rate limiting step in phosphatidylcholine synthesis, a major phospholipid in *Plasmodium*. Compounds such as this step could be effective against parasites resistant to existing antimalarials.

- Biochemical characterization of the pharmacological target, to find the causes of the hyperfunctioning of the choline carrier after malarial infection.
- Synthesis of new molecules among the 3 hyperactive families to optimize the structural requirements.
- Determination of the antimalarial activity of the compounds in a broad spectrum of resistant strains *in vitro* and *in vivo*, and preclinical evaluation against *P. falciparum* in monkeys.
- Sub-cellular localization of the drugs and studies on the bioavailability and metabolism of one or two radiolabelled lead compounds.
- Determination of the ease or difficulty by which parasites become resistant to the lead compounds *in vivo* and *in vitro* by drug pressure.
- Research into the possible activities of the compounds against the non-erythrocytic stage of *Plasmodium*.

Expected outcome

This project should provide insights into the nature and site of the choline transporter and thus for the creation of new molecules that will inhibit plasmodial phospholipid metabolism. The project further expects to establish the therapeutic doses needed to block the multiplication of *Plasmodia* and to further develop such drugs that have a maximum therapeutic index.

Results

Choline entry in the infected erythrocytes involves an asymmetric cyclic carrier whose functioning is hyperactivated after *Plasmodium* infection. Choline transport in the erythrocytes is not sodium-dependent nor stereospecific as demonstrated using methylcholine stereoisomers.

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Choline transport in the infected erythrocyte possesses characteristics quite distinct from that of the nervous system allowing discrimination between the antimalarial activity and a possible toxic effect.

The synthesis of choline analogues has focused on quaternary ammonium salts whose structures have been optimized leading to 10 compounds possessing IC50 ranging around 10^{-9} M and acting via an inhibition of *de novo* PC biosynthesis. In this quaternary ammonium series (first generation of active compounds), the lead compound G25 (IC50 = 0.6 nM) was highly efficient *in vitro* against 55 *P. falciparum* polypharmacoresistant isolates with an IC50 also in the nanomolar range. No cross resistance *in vitro* was observed between G25 and 5 other classical antimalarials (chloroquine, quinine, mefloquine, halofantrine and artemether). *In vitro*, G25 was also active on *P. berghei*, but IC50 was about 50-fold higher than on *P. falciparum*. It was very specific to mature parasites (trophozoites) with specific ultrastructural alterations of the endoplasmic reticulum as determined *in vitro* against *P. falciparum* and *P. berghei*, and also *in vivo* against *P. chabaudi*-infected mice. This specificity corresponds to the most intense phase of phospholipid biosynthesis activity during the parasite cycle, thus corroborating the mechanism of action.

When tested on the asexual stages of *P. berghei in vitro*, no quantitative effect of the drug was evident on the numbers of parasites that underwent exflagellation, or on the process of exflagellation and gamete release, and no morphological alterations upon the male or female gametes could be discerned. By contrast, between 10^{-7} and 10^{-8} M, a reduction in ookinete numbers (probably due to an enhanced fragility rather than to an inhibition of fertilization) and motility was observed, along with an inhibition in the clustering of pigment within the ookinete resulting in generalized morphological abnormalities.

The *in vivo* antimalarial activity (ED50) against *P. chabaudi* was 0.05 mg/kg, and a slightly lower ED50 was obtained with *P. vinckei petteri*, as assessed when the drug was intraperitoneally administered in the 4 day suppressive test. On the other hand, *P. berghei* was revealed as less sensitive (3 - to 20-fold, depending on the *P. berghei*-strain) *in vitro* when compared to *P. falciparum*. *P. yoelii* also appeared less susceptible to G25. In the infected mouse model, an intrinsically lower sensitivity of *P. berghei* (also demonstrated *in vitro*) or *P. yoelii* to G25 was demonstrated compared to *P. chabaudi* or *P. vinckei petteri*. This difference in activity could result from intrinsically lower sensitivity of the *P. berghei* strain to G25, from invasion preference for mature or immature red blood cells of every strain or from their degree of synchrony. *In vivo*, G25 was curative when administered twice a day subcutaneously to *P. chabaudi*-infected mice, provided the treatment lasted for at least 8 consecutive days. It succeeded in completely inhibiting parasitemias as high as 11.2% without any decrease in its therapeutic index (LD50/ED50), indicating that it is thus not necessary to increase doses to reduce parasitemia even for severe infections.

When administered intramuscularly twice a day at 0.2 mg/kg to *P. falciparum*-infected Aotus monkeys, G25 also fully succeeded in curing parasitemia as high as 20% revealing that it is as effective as sulfadoxine + pyrimethamine (alone or in combination with quinine). As low as 0.030mg/kg G25 were sufficient to completely clear parasitemia without recrudescence after 60 days as checked by PCR.

Keeping in mind that the Maximal Tolerable Dose for the monkey ranges around 1.5 mg/kg, one can assume that Therapeutic index in the *P. falciparum*-infected Aotus monkey is higher than 50, revealing the very high efficiency of G25 *in vivo* against *P. falciparum*. Development of this pharmacological model has thus been fully validated with the malaria-infected-mouse and -monkey models. Nevertheless, the very low permeability to the intestinal barrier hinders a further development of such compounds for a widespread

use in uncomplicated malaria. Transition to a human preclinical investigation has required the synthesis of molecules which would be better tolerated and absorbed. For this purpose, a new chemical strategy was developed.

New compounds which were bioisosteric analogues of the very potent leader compound G25 were thus synthesized to improve oral absorption and tolerance. In these series, the quaternary ammonium group present in G25 was replaced by a basic bioisosteric group, which is mainly protonated at physiological pH. Among them, 10 new molecules, were highly active *in vitro* against *P. falciparum* (IC50 in the nanomolar range) their antimalarial activity being achieved by a mechanism of action involving *de novo* PC biosynthesis inhibition. In these series, MS1 was designed as the lead compound of the second generation of active compounds (IC50 = 0.5 nM). They revealed better absorption (by 7 to 22-fold) and were also better tolerated (by 15 to 20 fold) than G25. They also possess *in vivo* antimalarial activity in the *P. vinckei petteri* system (therapeutic index was 6 at best).

This low *in vivo* antimalarial activity against *P. vinckei* or *P. berghei* results from a low susceptibility of the murine parasite (as already observed *in vitro* with *P. berghei*) rather than from unfavorable pharmacokinetics of these compounds (as demonstrated with G25 of MS1) as evidenced by *ex vivo* tests (performed *in vitro* against *P. falciparum* in the presence of the serum of mice to which drug was administered). Indeed, considering slow blood clearance (t1/2 elimination = 10 h), a relatively high value of CO (200-fold higher than *in vitro* IC50 against *P. falciparum* after administration at LD50/3) and linear pharmacokinetics, MS1 also revealed activity *in vivo* against *P. falciparum*-infected monkeys (as G25), suggesting that therapeutic index for MS1 could be higher than 50 even with only one daily dose and also in the oral mode. The next experiments will test this compound *in vivo* against *P. falciparum* in the infected Aotus monkey.

G25 and MS1, tested for genotoxicity using the Ames test, did not show any mutagenic activity (with or without metabolic activation). Additional compounds are currently being synthesized not to improve the antimalarial activity which is already good enough (nanomolar range) but rather to obtain compounds with better tolerance and oral absorption. Some of them already are potential candidates for preclinical studies that could be planned within the next 2 years.

Another part of the biochemical section was aimed at anticipating and circumventing possible resistances which could be set up by the parasite when the supply of choline is blocked by pharmacological interference. This notably consisted in evaluating rate limiting steps in crucial pathways and also in detecting potential vital pathways (absent from the host).

CTP: phosphocholine cytidylyltransferase (CCT) (EC 2.7.7.15) is the rate-limiting and regulatory enzyme in *de novo* PC synthesis.

The structural gene encoding for CCT was isolated from *P. falciparum* using PCR to amplify genomic DNA with degenerate primers constructed on the basis of conserved regions identified within yeast and rat liver CCT molecules, and using the PCR product to screen a genomic library. The *P. falciparum* CCT gene encodes a protein of 370 amino acids (42.6 kDa) and displays 41-43% homology to CCT molecules of the other organisms cloned to date. The central domain of CCT homologues, proposed as the catalytic domain of the CTP-transfer reaction, shows 68-72% homology among *P. falciparum*, human, rat liver, and yeast enzymes. This gene is present in a single copy and located on chromosome 3 of the parasite.

The molecular analysis of this gene will provide the mechanisms of transcriptional (or translational) regulation of the enzyme which regulates PC biosynthesis in *Plasmodium*. This will help to understand the mechanism of an eventual resistance of *Plasmodium* against original antimalarials acting by inhibition of this metabolic pathway.

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Contract number TS3*CT920145

IDENTIFICATION OF *PLASMODIUM FALCIPARUM* PROTEASES AND THEIR IMPLICATIONS FOR THE MEMBRANE ORGANIZATION THE INFECTED RED CELL. DESIGN OF HIGH AFFINITY RECEPTORS

Period: January 1, 1993 - December 31, 1995

Co-ordinator: MUSEUM NATIONAL D'HISTOIRE NATURELLE, LABORATOIRE DE BIOLOGIE PARASITAIRE ET CHIMIOTHERAPIE, Paris, France (J. SCHREVEL)

Objectives

- To study the proteinases of *P. falciparum*'s asexual stage, MPEI (Merozoite Proteinase for Erythrocyte Invasion), and Pf37, involved in the reinvasion process step of erythrocytes by merozoites.
- To study the cytoadherence of *P. falciparum* infected erythrocytes.

Activities

MPEI

MPEI active fraction was purified from schizont stages of *P. falciparum in vitro* cultures by its neutral cysteine protease activity, able to cleave the Gluconoyl-BLGK-AEC fluorogenic substrate. Several specific inhibitors, based on the VLGK peptidic sequence, were synthesised and tested on the *in vitro* invasion. They exhibited an IC50 at the mM level. Cloning of the MPEI gene was thus undertaken to provide material for determination of the three dimensional structure of the active site. A polyclonal antibody was raised against the purified MPEI fraction (containing proteins of 105, 97, 68 and 38 kDa), and used to screen two *P. falciparum* genomic expression libraries. Two novel single copy genes were isolated, partially sequenced and analyzed.

Pf37

The specific sites of cleavage of the human spectrin by Pf37 protease have been located within the different tryptic domains of the spectrin α-subunit, using polyclonal antibodies specific for the different domains. Recombinant peptides of different regions of the α-subunit were produced, and used in enzymatic assays with preparations of pre-purified Pf37.

Cytoadherence

The characterization of the proteins from the "virus-like" vesicles from *in vitro* cultured *P. falciparum* have been established by metabolic labeling with 35S-methionine, and Western blotting using polyclonal and monoclonal antibodies against membrane and cytoskeleton components of the erythrocyte.

The vesicles have been visualized by immunofluorescent probes and related to cyto-adherence as a function of the K+ or K- phenotype.

Results

Proteinases and erythrocyte invasion

MPEI

Partial sequences and analysis of the two cloned genes indicated that one (1626 bp open reading frame) encodes a protein with 31% asparagine residues, found in both repeated hexamers and in non-repeated regions. This gene corresponds to a novel asparagine-rich protein of *P. falciparum*. The second gene (1510 bp ORF) encodes a protein with highly significant homologies to Zn-metallopeptidases. The 27 amino-acids sequence of the active site unambiguously places it into the M1 family of Zn-metallopeptidase. This novel gene is the first cloned *P. falciparum* aminopeptidase.

Antibodies raised against recombinant forms, or synthetic peptides, of both homologous asparagine-rich and Zn-metallopeptidase molecules, yielded specific whole parasite labelling of the parasite in immunofluorescence assays. These antibodies recognize peptides migrating as the MPEI's in one dimensional SDS-PAGE, and immunoprecipitate the same peptides. More, aminopeptidases activities were found in schizont extracts: one of them co-purified with the MPEI cysteine protease.

Pf37

Pre-purified fractions of the Pf337 were shown to cut within two distinct regions of the α-subunit of human spectrin. One of the regions corresponds to the Src homology III (SH3) motif, with a secondary structure different from the rest of the polypeptide made of successive triple α helices. Enzymatic assays, using recombinant peptides of different segments of the human α-chain as substrate for Pf37, demonstrated that only recombinant SH3 was cleaved by the enzyme, at three close sites. Identification of the amino-acids at these cleavage sites is now in progress by determination of the precise mass of the hydrolyzed fragments by mass spectrometry and comparison with the theoretical masses deduced from the amino-acid sequence of the recombinant SH3. Furthermore, we could show that the SH3 motif from a non-erythroid spectrin, i.e. fodrin, was insensitive to the Plasmodium spectrinase activity. The results will allow us to design pseudo-peptides as specific inhibitors of the spectrinase activity.

Characterization of vesicle-like structures from P. falciparum in vitro cultures

The virus-like vesicles obtained from *in vitro P. falciparum* cultures were analyzed by comparison with normal red blood cell (RBC) cultures. The phenotypes of *P. falciparum* strains used were K+ (FcB1/Colombia) and K-(FcR3/The Gambia). To identify the parasite

origin of proteins, the infected RBC were radiolabelled with 35S-methionine and the erythrocyte membrane proteins were studied by Western blotting. The results were as follows:

⇒ Virus-like vesicles were observed *in vitro* in both normal and infected *P. falciparum* RBC cultures maintained under the classic conditions.

⇒ Parasite proteins were detected in the vesicles released from the *P. falciparum* infected RBC in contrast to the normal RBC.

⇒ The erythrocyte proteins of vesicles correspond to the intramembranous proteins (modified band II.1 glycophorin). All erythrocyte proteins associated with the cytoskeleton were observed (spectrin, ankyrin, protein 4.1 actin).

⇒ Up to now, no specific pattern of parasite proteins was associated with the K+ or K- phenotype of the strains.

Expected outcome

This approach may lead to the development of antimalarial drugs. Because numerous proteases activities have been shown to be essential for the survival of *Plasmodium falciparum*, our aim is to block specifically one or several of these activities, by using specific compounds structurally analogous to the substrates of the proteases. Such a strategy requires a detailed knowledge of the active site of the protease, in order to identify its best substrate. In this context, we have obtained promising results with protease inhibitors which can more easily reach their targets during host cell invasion, in contrast to intracellular proteinases involved in the haemoglobin breakdown.

Contract number TS3*CT920145

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Contract number TS3*CT930224

EPIDEMIOLOGY OF DRUG RESISTANCE IN *PLASMODIUM FALCIPARUM*

Period: October 1, 1993 - September 30, 1996

Co-ordinator: LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE,
DEPT. OF MEDICAL PARASITOLOGY
London, United Kingdom (D. WARHUST)

Objectives

- To evaluate drug sensitivity of *P. falciparum* malaria in 100 patients in the Prabies area of Guinea-Bissau and in the Banjarnegara area of Indonesia by the WHO *in vivo* (chloroquine) and *in vitro* tests (over the period 1993-95).

- To characterise the rRNA genes of the DNA samples obtained by probe and PCR studies.

- The training in malaria culture, drug-sensitivity testing, and molecular biological procedures of scientists from countries with endemic malaria, transfer of technology to their laboratories and to enhance their research potential.

Activities

* To collect dry blood spots and frozen blood samples from the patients for restriction and probe studies and polymerase chain reaction (PCR) investigation on drug resistance-related genes.

* To cryopreserve material for subsequent recovery into culture and to establish clones from selected cultured isolates, in particular, from up to 50 which have recrudesced after chloroquine treatment.

* To carry out restriction and PCR studies, and evaluation of drug sensitivities on the continuously cultured clones for comparison with results *in vivo* and *in vitro* on the fresh isolates.

Expected outcome

Workers from endemic areas will be trained in malariology, and the use of molecular and pharmacological approaches to study drug-resistance. There will be enhanced collaboration within and outside the EC in malariology. The expertise and understanding of all the collaborating countries will be enhanced.

Knowledge of the way in which malaria parasites become resistant will enable the development of imported and novel drug entities to attack malaria morbidity, reduce the population reservoir for the parasite's transmission and reduce the overall impact of this highly important vector borne disease.

Contract number TS3*CT930224

Valuable data on the drug response of *falciparum* malaria in the endemic areas concerned will also be obtained during the project, which will help in deciding the approach to malaria treatment in these areas.

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Contract number TS3*CT940297

DNA POLYMERASES OF MALARIA PARASITES: TARGETS OF CHOICE, BOTH FOR TREATMENT OF THE DISEASE AND FOR CONTROL OF THE INFECTION

Period: January 1, 1995 - December 31, 1996

Co-ordinator: INSTITUTE OF INFECTIOUS DISEASES AND IMMUNOLOGY,
DEPT. OF PARASITOLOGY & TROPICAL VETERINARY MEDICINE
Utrecht, The Netherlands (J.P. OVERDULVE)

Objectives

- To study DNA replication enzymes of malaria parasites as potential targets for new antimalarials and to identify, sequence and characterize their genes.
- To search for specific inhibitors of these enzymes as candidates of such anti-malarials, and to study their pharmacokinetics, target specificity and applicability.
- To study means - either by use of such inhibitors or by manipulation of the target genes - to lower the pathogenicity of a *Plasmodium* infection or the virulence of a *Plasmodium* strain so as to promote the chance of development of protective immunity by the host.
- To strengthen the capacity in this particular field of research in Thailand.

Activities

* Synthesis of a number of derivatives of the potent (but rather toxic) anti-plasmodial nucleotide analog HPMPA as well as their diphosphates (analogs of HPMP App, the intracellularly phosphorylated and presumed actual inhibitor).
* Determination of the inhibitory effect of these derivatives against malaria parasites (*P. falciparum*) in culture. If active, the inhibitory effect will be examined against *P. berghei* and/or *P. vinckei* in mice. The activity of diphosphate derivatives will be assessed against a number of plasmodial replication enzymes *in vitro*. These plasmodial replication enzymes will be produced as native enzymes by purification from parasite cultures and/or as heterologous expression products of their genes.
* Study of pharmacokinetics and metabolism of compounds that show antimalarial activity by HPLC analysis, and identification of the active metabolite(s) and the actual target(s).
* Identification of the enzyme responsible for intracellular phosphorylation to the diphosphate form and analysis of the kinetics of this activation of HPMPA and congeners.

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As a corollary of this research investigations will be started to induce protective immunity in rodents by parasites "attenuated" either by direct use of plasmodial replication inhibitors or by manipulation of target enzyme genes.

Expected outcome

⇒ Identification of the target(s) and mechanisms(s) of action of (deaza) HPMPA and related compounds.

⇒ Sufficient insight into structure/function relationship of this class of putative replication inhibitors to allow a rational search for and future synthesis of effective and suitable antiplasmodial inhibitor(s).

⇒ Increased knowledge of the replication machinery of malaria parasites and its underlying genes to permit manipulation of this machinery in order to decrease malaria pathogenicity and/or parasite virulence.

⇒ Strengthening of the research capacity in this particular field of research of the Thai research partner so as to increase their role as a center of excellence for malaria research in the South Asian Region.

Results so far

Target and mechanism of action of HPMPA

The targets of HPMPA at the parasite stage level are asexually multiplying forms (schizonts) in erythrocytes and - even more sensitive - liver cells. Microgametogenesis and meiosis in ookinetes are insensitive.

Liver stages of *P. berghei* in mice are all killed by sustained release of a total dose of only 0.45mg/mouse during the first two days of sporozoite-induced infection. Recovered mice were partially immune against sporozoite challenge: survival time increased by a factor of about 2.5 and cerebral malaria, normally causing death within 9 days in control animals was absent.

In erythrocytes the effect of HPMPA is apparent at the late schizogony stage: schizonts are arrested at about the 6-nucleated stage.

At the molecular level pharmacokinetics studies, using HPLC, on *P. falciparum* cultures of wild-type and resistant mutants incubated with HPMPA showed that HPMPA was incorporated into the DNA at a ratio of about 1 in 20,000 and 1 in 70,000 adenosines, respectively, with 60% of the incorporated HPMPA label consisting of HPMPAp, suggesting defective DNA repair caused by HPMPA and interruption of DNA synthesis after incorporation of HPMPA.

Molecular studies on mutant clones of *P. falciparum* with different levels of resistance to HPMPA focusing on the possible role of DNA polymerases as targets of HPMPA, revealed that resistance is associated with a single mutation at a specific site.

This mutation results in a substitution of a valine which is highly conserved in DNA polymerase δ enzymes of different eukaryotic origin as well as in herpes virus homologues. It is located in the 3'-5' exonuclease, proof-reading domain in the N-terminal half of the DNA polymerase δ and in the DNA polymerase α gene, which are both responsible for DNA polymerase activity.

Three other independent clones of a total of 16, with resistance up to 12 times the wild-type sensitivity, showed a single mutation at the same site when 543 nucleotides around this site were sequenced.

From the location of the substitution we conclude that HPMPA-induced arrest of DNA replication does not result from direct inhibition of the polymerase activity of the enzyme. Instead, incorporation of HPMPA, inducing e.g. chain termination or distortion of the DNA, can be considered as its mode of action, and increased proof-reading capacity of the DNA polymerase δ exonuclease activity by substitution of a highly conserved valine as inducing resistance to HPMPA.

Structure/function relationship of nucleos(t)ide analogs

A total of 68 analogs were investigated for anti-plasmodial activity in *P. falciparum* cultures. From the results it can be concluded that 3 chemical entities seem to be imperative for anti-plasmodial activity: a purine base, a hydroxyl group in the acyclic side chain, and a phosphonate group terminating this chain.

Genes of the replication machinery of malaria parasites

The 49 kDa primase subunit of the DNA polymerase α complex, sequenced previously, has been expressed in large quantities as a fusion product with an N-terminal His-tag. An indirect primase activity assay with the purified renaturated enzyme showed that this subunit alone is able to synthesize RNA precursors *de novo*, in contrast to the mouse homologue which has been reported to be only active after heterodimer formation with the 58kDa subunit.

A similar approach has been followed with respect to the C-terminal polymerase domain of the DNA polymerase α gene, with similar results except that enzymatic activity has not yet been established. Also constructs containing the N-terminal exonuclease domain of the pol α gene, with and without an N-terminal His-tag, have been made, as well as a construct containing the whole pol δ gene without a His-tag. Expression of the whole pol δ gene has not yet yielded positive results and is now studied at the mRNA level.

The whole topoisomerase II gene after sequencing has been inserted in a Bacoluvirus transfer vector and expression experiments have been started.

The DNA polymerase α enzyme of *P. falciparum* has been further purified by FPLC and micro N-terminal amino acid sequencing of the putative enzyme, a purification product of 150kDa (SDS-PAGE), has been performed.

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Strengthening of the research capacity of the DC research partner

A second PhD student from the DC partner's laboratory has, after a training period of almost 2 years in the laboratory of the coordinator, returned to her home laboratory and is continuing plasmodial DNA polymerase and topoisomerase research there.

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Contract number IC18*CT960056

PHOSPHOLIPID METABOLISM, A NOVEL TARGET FOR ANTIMALARIAL DRUGS: DEVELOPMENT OF THE PHARMACOLOGICAL MODEL

Period: October 1, 1996 - October 1, 1999

Co-ordinator: UNIVERSITE MONTPELLIER II, DEPT. BIOLOGIE ET SANTE
Montpellier, France (J. SCHREVEL)

Objectives

The main objective of the project is the development of new antimalarial drugs that interact with the malarial parasite phospholipid metabolism and could provide a solution to *P. falciparum* polychemoresistant malaria.

Although there is no indication of potential resistance to date, we believe that we must actively initiate studies concerning mechanisms that could be involved in potential acquisition of resistance to the effectors. This molecular approach is worth immediate investigation since precise mechanisms can be expected from the suspected drug site. Results could lead to the knowledge of a whole set of metabolic pathways vital for parasite growth.

The main objectives will be to synthesize new series of original and potentially alternative compounds aimed at improving tolerance and oral absorption. Identification, isolation and characterization of the pharmacological target.

To carry out thorough antimalarial activity studies including chemosensitivity, therapeutic index after *in vivo* oral formulations in *P. falciparum*-infected monkeys, and against others stages (non erythrocytic) or species (e.g. *P. vivax*).

To describe the pharmacokinetic properties and toxic evaluation of lead compounds will also be studied.

Experimental induction of resistances, characterisation of effector-resistant *P. falciparum* malaria and alternative to resistance. In case of resistance, combinations with other current approaches would be studied.

Activities

* Chemical synthesis of compounds aimed at improving tolerance and oral absorption. This includes compounds with new cationic heads that could be used if the current lead compounds may prove to have unacceptable drawbacks, and also the synthesis of prodrugs with the aim of improving oral absorption and, eventually to promote the development a new generation of effectors.

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* Identification and characterisation of the pharmacological target. Affinity chromatography using a column of immobilized effectors is proposed. If necessary, chemists will also synthesise photoreactive lead compound derivatives. Pharmacological target cloning should allow its complete characterisation, determination of the active site and help in the design of new effectors.

* It is of utmost interest to study the mechanisms of regulation of PL biosynthesis pathways in Plasmodium. More than just a problem of metabolic regulation, this program concerns mechanisms which could be involved in resistance that the parasite could develop when the supply of choline is blocked due to pharmacological interference. Biochemical and genetical approaches will be used as a powerful tool for the elucidation of metabolic regulations as well as the biological significance of the different metabolic pathways in Plasmodium. We will be particularly concerned by the metabolites and activities of CDP-choline pathway which synthesizes de novo PC. Additionally, we will focus on PS Decarboxylase activity which also provides Plasmodium for an important part of PC.

* Antimalarial activity, pharmacokinetics and toxic evaluation of lead compounds. The first priority tasks will be (1) in vitro and in vivo evaluation of antimalarial activity against *P. falciparum* blood stages. (2) 4 to 5 lead compounds will be tested in the *P. falciparum* / SCID mouse model , (3) therapeutic index of various formulations (intramuscular and oral modes) of 2-3 compounds in Aotus monkeys infected with P. falciparum. Blood samples will also be collected to perform bio-assays of the seric compounds.

* Activity of 2-3 lead compounds against *P. falciparum* isolates with various degrees of resistance will be determined.

* To evaluate antimalarial activity against *P. vivax*/*P. cynomolgi* blood stages. (2-4 lead compounds). According to results, to evaluate against *P. cynomolgi* in rhesus monkey and against *P. vivax* in Saimiri monkey for comparison with *P. falciparum*/Aotus results.

* To test the activity of the lead compounds against the non-erythrocytic stages of Plasmodium.

* To determine pharmacokinetics properties of lead compounds (ex vivo tests), and to determine toxicity of lead compounds.

* Resistance mechanism and alternative to resistance. This includes the in vitro induction of resistance against choline analogs, the characterisation of effector-resistant *P. falciparum* malaria (pharmacological target and lipid metabolism), and examination of genes associated with resistance to standard antimalarial drugs Alternatives to resistance would include combined action of PL metabolism inhibitors with known antimalarial drugs.

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Expected outcome

The work content is totally devoted to the establishment of a new pharmacological model. Although the outcome of fundamental and experimental research can never be known in advance, the numerous complementary experimental approaches that are planned within the different partner laboratories, should allow to reach the proposed objectives. It is anticipated that some of our research effort will lead to potential industrial or pharmacological outcomes.

Concerning malaria, by now, the most urgent need concerns a first-line oral substitute to chloroquine. That is one of the reasons we want to achieve an oral formulation of our compounds, rather than risking the development of a non-oral administerable compound.

The target could be a common one between different parasites. However, until now, significant inhibition at concentrations lower that 1 mg/l has not been observed for any other parasites except *Babesia*. On the other hand, the susceptibility of the protozoan parasite *Babesia*, that also invades erythrocytes but is not sensitive to haemoglobin degradation-related lysomotropic agents, is interesting as it confirms the absence of cross-resistance of the PL metabolism pharmacological effectors with the current lysomotropic antimalarial agents.

Contract number IC18*CT960056

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Contract number IC18*CT960074

DEVELOPMENT OF NOVEL DRUGS AGAINST MALARIA

Period: November 1, 1996 - October 31, 1999

Co-ordinator: STATENS SERUM INSTITUT, DEPT. OF CLINICAL MICROBIOLOGY, Copenhagen, Denmark (A. KHARAZMI)

Objectives

The general objective of this research proposal is to select an appropriate candidate compound from a group of oxygenated chalcones with novel activity against malaria parasites for further development into a new antimalarial drug.

The specific objectives are:

- To develop an optimal method for the synthesis of necessary amounts of the appropriate compounds.
- To determine the purity, physicochemical properties and stability of these compounds.
- To examine the *in vitro* activity of these compounds against different strains at erythrocytic and liver stages of human malaria parasite *Plasmodium falciparum*.
- To investigate the structure-activity relationship of these compounds.
- To test the activity of selected compounds on the parasite isolates from the blood samples from malaria patients in Colombia.
- To carry out *in vivo* studies to determine the most appropriate formulation for administration of these compounds.
- To select the most appropriate compound(s), based on their *in vitro* activity and test them against *P. berghei* and *P. yoelii* in murine models and against *P. falciparum* in Aotus monkeys.
- To investigate the mechanism of action of these compounds on parasite mitochondria.
- To perform studies on absorption, tissue distribution, pharmacokinetics, and pharmacodynamics in rats, dogs and monkeys.

Activities

* Development of optimal synthesis methods. A study taking advantage of the methodology developed for Quantitative Structure-Activity Relationships (QSAR) will be utilized to disclose the optimum structure for the antimalarial activity. Based on the outcome of the QSAR analysis new types of compounds will be prepared and evaluated. A procedure for large scale synthesis of the selected compounds will be developed.
* *In vitro* studies against blood stage *Plasmodium falciparum*. The effect of a wide range of compounds on different chloroquine sensitive and resistant strains of *P. falciparum* will be tested using conventional methods. Fifty *P. falciparum* isolates collected in an endemic region of Colombia will also be studied.
* *In vivo* studies in murine models. The appropriate compound(s) for the *in vivo* studies will be selected based on their *in vitro* activity against *P. falciparum*. Several mouse models of *P. yoelii* and *P. berghei* infection will be used in these studies. The compounds will be administered by different routes and dosages and at different time periods to infected animals. Levels of parasitemia in Giemsa-stained smears and survival of animals will be assessed.
* Studies on the liver stage of *P. falciparum*. The activity against pre-erythrocytic stages will be tested using *in vitro* assays with different *P. falciparum* and *in vivo* in mice with *P. yoelii* or *P. berghei*. The *in vivo* studies will include i.v. inoculation of sporozoites into mice. The effect of various compounds will be examined by either i.p. or oral administration of selected compounds.

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* *In vivo* studies in Aotus monkeys. The monkeys will be infected and once parasitemia reaches 1.0% they will be treated with the test compound by giving an oral dose of each compound for different time periods. Parasitemia will be determined daily by thick smears and whenever necessary by PCR.
* Studies on the mechanism of action. The effect of these compounds on the ultrastructure by EM and on the function of mitochondria will be carried out. The effect of these compounds on some of the enzymes of the respiratory chain in mitochondria such as fumarate reductase will also be examined.
* Formulation studies. These studies will include preparations based on aqueous solutions by addition of solubility promoting substances e.g. polyalcohol, glycolether and glycolesters or solubilizing agents. Investigation of preparations based on particulate drug delivery systems e.g. oil/water emulsion will also be undertaken.

Expected outcome

The most important expected outcome of this study will be identification of an appropriate oxygenated chalcone for phase 1 studies.

⇒ This study will demonstrate the structural requirements for optimal selectivity and activity for chalcones as antiprotozoal drugs.
⇒ This study will show the activity of these compounds against different strains of *P. falciparum in vitro* and their activity against different species of *Plasmodium* in several animal models.
⇒ This study will provide insights into the mechanism of action of the selected compound(s).
⇒ This study will identify the appropriate formulation for *in vivo* administration of the selected compound(s)

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Contract number IC18*CT970223

DEVELOPMENT OF INHIBITORS OF MALARIAL AND TRYPANOSOMAL DIHYDROFOLATE REDUCTASE AS ANTIPARASITIC AGENTS THROUGH COMBINATORIAL CHEMISTRY

Period: October 1, 1997 - September 30, 2000

Co-ordinator: NATIONAL SCIENCE AND TECHNOLOGY DEVELOPMENT AGENCY, Bangkok, Thailand (Y. YUTHAVONG)

Objectives

- To test the validity of the combinatorial synthesis approach to antiparasitic drug development, based on the inhibition of the parasite dihydrofolate reductase domain of the bifunctional enzyme dihydrofolate reductase-thymidylate synthase (DHFR-TS), and to develop new drug candidates especially against malaria from this approach.

- To understand the relation between structure and function of the parasitic enzymes, the basis for differential inhibition of the enzymes by candidate agents, and the basis for development of resistance.

Activities

Selected libraries of 2,4-diaminopyrimidines and dihydrotriazines which include known inhibitors of malarial dihydrofolate reductase will be created and screened against recombinant parasite and host enzymes. Libraries will be made both in solution and on solid supports. The second type of libraries will also be encoded with appropriate molecular tags for easy identification of structures. In the first type of libraries, screening will be made against the enzyme, either in free form or linked to an appropriate solid support, while in the second type, screening of the resin-bound libraries will be made against the enzyme linked to an appropriate fluorescent molecule.

Enzyme-bound drug candidates can be separated from the other compounds by centrifugal dialysis after addition of the enzyme. Alternatively, resin-bound enzyme can be used to trap the drug candidates. Identification of bound inhibitors from the first type of libraries will be made after extraction into organic solvent by high-performance liquid chromatography coupled with mass spectrometry and other analytical methods. Identification of bound inhibitors from the second type of libraries will be made, using a confocal laser microscope with a micromanipulator and an adapted fluorescent activated cell sorter.

After identification of these compounds, they can be synthesized in solution in bulk for further studies. This is important in order to ensure that the binding observed in the screening was not influenced by linking to solid supports or synergistic or antagonistic interaction of the molecules in the mixture. The characteristics of enzyme inhibition will be studied in solution to ensure that tight-binding components are indeed good inhibitors of enzyme activity. Emphasis will be made on discovery of compounds which effectively inhibit mutant enzymes from drug-resistant parasites.

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Testing of inhibition of *P. falciparum* in culture will also be done as well as testing of enzyme inhibition. After identification of promising candidate molecules, focused sublibraries will be made to cover various structural possibilities related to the identified molecules. In parallel, molecular modelling of the dihydrofolate reductase of the malarial and trypanosomal enzyme will be made, using information from known structures of the Leishmanial enzyme as well as bacterial and mammalian enzymes. Site-directed mutagenesis of the malarial enzyme will allow us to create a number of enzymes with mutations at sites that are suspected to be important for binding of substrates and inhibitors. This will allow us to establish a model of binding between the inhibitors and the enzyme, further allowing optimisation of the inhibitor structures to obtain better drug candidates for both the wild-type enzyme and the mutant enzymes arising from resistance.

Expected outcome

⇒ The validity of using combinatorial libraries for development of antifolate antimalarials will be tested.

⇒ Combinatorial libraries based on 2,4-diaminopyrimidines and dihydrotriazines in solution or on solid supports will be made, as will milligram quantities of recombinant wild-type and mutant malarial dihydrofolate reductases ready for screening.

⇒ Appropriate screening methodologies for combinatorial libraries against the malarial enzyme will be established.

⇒ Better understanding of inhibitor binding to the enzyme will be achieved.

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Contract number ERBIC18CT970227

DETECTION OF PLASMODIUM FALCIPARUM RESISTANT TO ANTIFOLATE AND SULPHONAMIDE DRUGS, AND ASSESSMENT OF THE EFFICACY OF DRUG TREATMENT USING MOLECULAR METHODS

Period: November 1, 1997 - April 30, 2001

Co-ordinator: ROYAL TROPICAL INSTITUTE,
Amsterdam, The Netherlands (L. OSKAM)

Objectives

To evaluate molecular methods for the detection of antifolate and sulphonamide resistant malaria parasites, and to apply these methods to an epidemiological investigation of the prevalence of genes conferring resistance in populations of *Plasmodium falciparum*. The ultimate aim of the work is to contribute towards a rational use of such drugs in control strategies against the disease.

Activities

* To evaluate the sensitivity and specificity of a molecular assay - mutation specific polymerase chain reaction (MS-PCR) and *in situ* PCR - for the detection of antifolate (pyrimethamine) and sulphonamide (sulfadoxine) resistant mutants of *Plasmodium falciparum.*
* To evaluate the technique of semi-quantitative nucleic acid sequence based amplification (Q NASBA) for the detection of low levels of malaria parasites and for the determining of the efficacy of drug-treatment.
* To apply these assays for epidemiological monitoring of drug-resistant mutants of *P. falciparum* in different drug-treatment regimens by parasitological and clinical criteria and by the analysis of the kinetics and degree of parasite clearance by NASBA.
* To increase and improve the local research capacity by providing training in molecular detection methods for the epidemiological and diagnostic investigation of drug-resistance of *P. falciparum.*

Expected outcome

The results will show the prevalence of antifolate and sulfadoxine resistant malaria parasites in malaria patients in Kenya and Zimbabwe. The prevalence of resistant strains is expected to be high in Kenya and low in Zimbabwe. In Kenya, MS-PCR will be applied on samples collected during the second and third year of the study and in Zimbabwe the method will be applied on samples collected in the first, second and third year. This will show whether the prevalence changes in times and whether chains harbouring different mutations will arise.

In situ PCR will provide insight in the prevalence of mixed infections.

The NASBA results will provide information on the treatment efficacy of antifolates and sulphonamides.

Information will be obtained on the relative sensitivities of the different assays in determining drug-treayment efficacy.

Contract number ERBIC18CT970227

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Contract number TS3*CT920070

COMMUNITY BASED MALARIA CONTROL UNDER THE GUIDANCE OF HEALTH SERVICES: INTERVENTION STUDY IN ECUADOR AND COLOMBIA

Period: January 1, 1993 - August 31, 1994

Co-ordinator: UNIVERSITÄT HEIDELBERG, INST. FÜR TROPENHYGIENE & OEFF. GESUNDHEITSWESEN, Heidelberg, Germany (A. KROEGER)

Objectives

- To describe the epidemiology of malaria in the study areas.
- To measure the impact of several community based interventions of malaria control on the incidence of malaria attacks and on case management.
- To identify factors which favour or hinder the protective efficacy of specific interventions.
- To measure different cost aspects of such a programme

Activities

* After the baseline study on malaria incidence and a KAP study in 3 study areas of Ecuador and Colombia regarding self-diagnosis and self-treatment of malaria, the study communities were divided randomly into intervention communities and control communities respectively (randomised community trial).
* Training of trainers: the project teams trained staff members of the local health services. They learned how to carry out workshops with health-promoters and volunteers of the malarial control programmes and how to use the training materials. All aspects of community based malaria control were dealt with, with special emphasis on rational use of antimalarial drugs, protection with mosquito nets as well as draining of water from breeding places. The newly trained staff carried out a series of training workshops in the intervention areas with community representatives and community volunteers. The research team observed these activities and established a mapping of vector breeding places as well as a regular counting of larval densities.
* After 8 months of intervention the research team repeated the measurements again both in the intervention and control areas assessing the malaria incidence and people's KAP regarding malaria prevention and treatment.
* Before and after the intervention, health services' staff (particularly malaria field workers) were observed when carrying out workshops and when doing community health actions, particularly DDT residual spraying.
* During the activities described above, costs were monitored to enable calculation of the overall costs of the programme.

Results

Between the 3 study areas on the Pacific Coast (2 in the Esmeraldas Province of Ecuador, 1 in the Chocs of Colombia) there was a considerable variation concerning socio-economic characteristics of the populations, accessibility of health services and ethnomedical practices. The demographic structure, housing and malaria control through vertical programmes were similar. Main malaria vectors were:

* *A. albinaus* in Ecuador and *A. nuneztovari* in Colombia. The monthly incidence rates of malaria episodes during the wet season was 3.5% in Columbia and 7.0% in Ecuador. The main parasite was *P. falciparum* (92% in Colombia, 86% in the North Coast of Ecuador). The remainder was *P. vivax*. Transmission occurred principally inside or around the houses. Users of impregnated bed nets had the same incidence of malaria episodes as non-users of bed nets.

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* The impregnation of bed nets with lambdacyhalothrin showed a high (71%) protective efficacy against clinical malaria attacks in Colombia. The study in Borbon (North Coast of Ecuador) showed that intensive residual spraying with DDT had the same protective efficacy against malaria episodes as the impregnation of bed nets with permethrin; however, the costs of the DDT programme were 3.5 times higher than those of the impregnation programme. It was observed that residual spraying activities estrange malaria workers from the communities while bed net impregnation enhances joint community actions and improves the communication between health services and local dwellers. In Mulsne (Ecuador) the protective efficacy of bed net impregnation was not enhanced by the additional breeding of larvivorous fish in all large mosquito breeding places.

A set of limiting and favourable factors for a community based programme of bed net impregnation was identified.

The educational programme in Ecuador had the following impact (expressed as % increase of correct knowledge and practice): improvement of correct knowledge about malaria transmission (30%), symptoms (25%) and the correct doses of chloroquine for adults (25%); the respective values in Colombia were 28%, 38% and 48%. The correct doses of chloroquine taken during a clinical malaria attack was improved by 20% in Ecuador and by 46% in Colombia. The factors related to it were analyzed.

The community interventions on mosquito breeding places had no clear effect on vector densities and malaria incidence rates, mainly because it was not possible to drain and fill them up all and/or because Anopheles mosquitoes flew in from the surrounding areas.

The cost calculations showed that if is feasible to carry out an impregnation programme on a large scale.

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Contract number TS3*CT920083

THE CONSEQUENCES OF MALARIAL INFECTION IN PREGNANT WOMEN AND THEIR INFANTS

Period: January 1, 1993 - December 31, 1995

Co-ordinator: LIVERPOOL SCHOOL OF TROPICAL MEDICINE
Liverpool, United Kingdom (B. BRABIN)

This research proposal had the general aim of describing the prevalence and pattern of malaria and anaemia in mothers and their infants in a rural area of Malawi where malaria transmission is endemic, in order to establish a basis for developing sustainable interventions which would be targeted at those in most need.

Objectives

- To quantify the prevalence and pattern of malaria and anaemia in mothers and their infants living in a malaria endemic area.
- To investigate whether birth haemoglobin relates to the development of anaemia in infants.
- To investigate the association between fetal anaemia, maternal iron status and malaria in pregnancy.
- To quantify the contribution of maternal anaemia and iron status to fetal growth retardation and low birthweight.

Additional objectives related to a technical amendment were:

- To determine the HIV status of recruited women.
- To determine the HIV status of babies recruited in the cohort study and born to mothers in the Malaria Pregnancy study.

This work gave a high priority to training components for young scientists and laboratory technicians from Malawi and Europe.

Activities

Case control study

Pregnant women attending the selected health centers were screened, after informed consent, at their first and further antenatal visits and at delivery. The women were enrolled at random and were matched for parity and week of delivery.

The women were screened for haemoglobin, hematocrit free erythrocyte protoporphyrin and haemoglobin electrophoresis. A blood film was made regularly for hematology and malaria microscopy. Cord blood obtained at delivery was screened for haemoglobin and malaria. The placenta was weighed and a smear was taken for malaria. From the newborn, birthweight, length and heel circumference were measured and the Ballard score was used to assess the gestational age of the newborn. The HIV status and vitamin A status of the mother was assessed.

Linear regression analysis was used to identify iron status, anaemia or malaria experienced during pregnancy, as a risk factor for low birth haemoglobin, low birthweight or fetal growth retardation.

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Cohort study

From the case control study information was obtained to identify cohorts for the longitudinal study. The cohort was selected by cord haemoglobin forming one group with an Hb >14 g/dl and a control group with an Hb >16g/dl. Babies were matched by sex, birthweight and maternal parity and were enrolled throughout the wet and dry season.

Morbidity surveys were completed monthly to assess illness episodes in the two weeks prior to each study visit. A blood film for malaria microscopy and for haemoglobin estimation was taken at 2, 4, 6, 9 and 12 months. At 2, 6 and 12 months infants were assessed medically for growth and haematologically for hematocrit and free erythrocyte protoporphyrin and a malaria smear was made. Hb electrophoresis were done on the 6 or 12 month samples. Sera was also screened for HIV seropositivity.

Results

The prevalence of *P. falciparum* malaria was high in the Shire Valley and the current policy of promoting 1 routine dose of sulphadoxine-pyrimethamine during pregnancy for malaria control was not significantly reducing parasitemia at delivery, especially in the wet season. Cord haemoglobin values indicated that fetal anaemia was a common problem and related to maternal haemoglobin at delivery and was affected by seasonal changes. Cord haemoglobin was related to placental parasite density and associated with an increased prevalence of infant anaemia as well as increased infant mortality. The HIV status of women was significantly associated with malaria prevalence in pregnancy and reduced the protection provided by parity specific immunity. Infant mortality was 3 times higher for babies of HIV+ women and 6 times higher if these mothers also had malaria at booking. This increased risk occurred despite evidence that vertical transmission of HIV was not increased with maternal malaria. Malaria in pregnancy was associated with significantly increased risk of infant mortality in HIV+ mothers. The high level of illiteracy in this population indicated a need to develop health interventions for malaria control in pregnancy which are based on literacy programmes since illiterate women were less likely to use antenatal care facilities. Maternal anaemia at delivery was significantly associated with low birthweight in primiparae and multiparae with a two-fold increase in low birthweight in first pregnancies if mothers were anaemic and up to a four-fold increase with severe maternal anaemia. Analysis of how this is confounded by other maternal characteristics is on-going.

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Contract number TS3*CT930225

EPIDEMIOLOGY AND CONTROL OF MALARIA IN THE PROVINCE OF RATCHABURI, THAILAND

Period: April 1, 1994 - September 30, 1996

Co-ordinator: ASSOCIATION SANTE SUD, DIVISION DES PROJETS
Marseille, France (M. SALICETTI)

Objectives

- Through an epidemiological study to determine the behavioral and environmental factors which influence the transmission of malaria in the village in the province of Ratchaburi.
- To measure the prevalence, distribution and seasonal variations in the incidence of malaria infection in 1000 inhabitants of 7 hamlets of a village in the district of Suam Phung.
- To evaluate the efficacy of the recommended anti-malarial treatments.
- To identify the *Anopheline* species in the village and to try to establish a relationship between entomological parameters and malariometric indices.
- To define and validate new parasitological diagnostic methods.
- To evaluate the clinical, parasitological and entomological impacts of the use of deltamethrin impregnated bednets.

Activities

The preliminary phase of the work (April - June 1991) consisted of creating a team which completed a demographic survey of the hamlets of the village and installed a health center which would carry out the epidemiological studies and diagnose cases of malaria. At the same time a questionnaire on the behavior of the population was administered so that the consequences for malaria infection could be examined.

The second phase of the work (July 1994 - June 1995) was a period of monitoring and characterization of endemic malaria in the population before the third phase, (July 1995 - June 1996) when deltamethrin impregnated mosquito nets were provided to 450 inhabitants, the whole population of hamlet G and to most of the population of two other hamlets (A and B). During these two phases blood samples were taken for thick and thin smear preparation from all 1000 inhabitants for active case detection. The same parasitological examinations were made for any febrile illness. In some cases the GBC® and PARASIGHT® diagnostic tests were used.

Surveillance after treatment with mefloquine (750 mg in a single dose) for *P. falciparum* or with chloroquine (1500mg chloroquine for 3 days and 15 mg primaquine per day for 14 days) for *P. vivax* as continued for 14 days.

Tests for chemoresistance of *P. falciparum* were carried out in 1994, 1995 and 1996 by the Rieckmann test and a radio-immunoassay.

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Entomological studies were completed each month (including vectorial capacity, parity, exophily, endophily).

Results

The preliminary results from phase two indicated a mean incidence of 1.9 episodes. 1000 inhabitants per day or 693.5 episodes/1000 inhabitants per year. There was variation among the hamlets and incidence was highest during the rains, from May to September. Males had twice as many episodes as females (912.5 episodes/1000 men per annum versus 474.5 episodes/1000 women per annum). Four species of malaria were described *P. falciparum* (48%) *P. vivax* (45%) *P. malariae* (6.2%) and *P. ovale* (0.8%). Chloroquine resistance of *P. falciparum in vitro* was evaluated in 1994 and 1995 as 100% (6 isolates tested) and 46.4% (28 isolates tested). Tests for resistance to quinine and mefloquine were carried out at the same time. No resistance was detected.

The efficacy of mefloquine was confirmed *in vitro* by examination of 10% of post-treatment recrudescences. *P. vivax* showed no *in vitro* resistance. Entomological studies revealed the presence of *A. durus*, *A. minimus* and *A. maculatus*.

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THREE YEARS CONTROLLED TRIAL OF LAMBDA-CYHALOTHRIN IMPREGNATED BED NETS AND MALOPRIM/PLACEBO CHEMOSUPPRESSION IN CONTROL OF MALARIA IN CHILDREN LIVING IN A HOLOENDEMIC AREA OF SIERRA LEONE

Period: January 1, 1994 - December 31, 1995

Co-ordinator: STATENS SERUMINSTITUT, DIVISION OF BIOTECHNOLOGY, LAB. OF PARASITOLOGY, Copenhagen, Denmark (E. PETERSEN)

Objectives

- Determine the efficacy of lambda-cyhalothrin impregnated bed nets and dapsone/pyrimethamine fortnightly chemosuppression, singly and in combination on malaria morbidity in children living under stable continuous malaria transmission.
- Determine the impact on the Anopheles population of village wide application of impregnated bed nets.

Summary

* Most previous intervention studies have looked only at the effect of the intervention over no more than a 12 month period, which is not sufficient to demonstrate any long term effect on the population.
* There was a clear difference between the dapsone/pyrimethamine singly and combined with impregnated mosquito nets during the first 12 months of the study, but this difference disappeared during the second intervention year and first half of the third year.
* Malaria episodes per 1000 child weeks at risk was in the first and second intervention year in the control group 37.7 and 49.9 respectively; in the group receiving impregnated bed nets alone 19.2 and 18.7; in the group receiving fortnightly dapsone/pyrimethamine 22.2 and 41.4 and in the group receiving both impregnated bed nets and fortnightly dapsone/pyrimethamine 10.7 and 14.8.
* This gave protective efficacy relative to the control group of the lambda-cyhalothrin impregnated bed nets alone of 49% and 62% in the first and second intervention year; of fortnightly dapsone/pyrimethamine of 42% and 18% in the first and second intervention year and in the group receiving both impregnated bed nets and fortnightly dapsone/pyrimethamine of 72% and 70% in the first and second intervention year respectively.
* The decreased efficacy of dapsone/pyrimethamine alone and the minimal difference in protective efficacy between the net and net plus dapsone/pyrimethamine strongly suggests that dapsone/pyrimethamine was much less effective during the second year. The only explanation for this decrease in efficacy is an increase in drug resistance to dapsone, pyrimethamine or both.
* The development of resistance to dapsone, pyrimethamine or both was confirmed by *in vivo* susceptibility testing performed pre-intervention and post-intervention. The initial response to a prophylactic dose of dapsone/pyrimethamine was the same in 1992

(pre-intervention) and 1995 (post intervention) measured by the parasite rate 7 days after drug administration. However, the long lasting reduction in parasitemia had almost disappeared in 1995, when parasite rates were back to initial values after 2 weeks compared to 6 weeks in the pre-intervention study.

* Comparing the geometric mean parasitemia in children who remained parasite positive at each examination during the *in vivo* assay, children who in 1992 were parasite positive at day 2 had a very low parasite density, which increased to background level at day 14. In 1995, there was no significant change in parasite density after drug administration, showing that the reduction was achieved in 1992 in children parasite positive at day 7 had vanished after three years of continuous dapsone/pyrimethamine administration.
* Sickle cell trait, HbAS, was found in 18.3% HbF in 1.8% and HvC in 0.9% of the children.
* A higher proportion of blood-fed Anopheline mosquitoes were caught resting in net villages, which suggests that the presence of the nets in the rooms drives away blood-fed mosquitoes to their outdoor resting site.
* We found no evidence of decreased susceptibility to lambda-cyhalothrin during the study period.
* Impregnated bed nets significantly reduced indoor resting density of, and exit trap collections of Anopheline mosquitoes, but did not have any effect on human bait catching and light traps catching.
* The parity rates of Anopheline mosquitoes were significantly reduced in villages with impregnated nets.

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Contract number IC18*CT970210

DELAYED CHILD MORTALITY AND INSECTICIDE-IMPREGNATED MATERIALS: THEORETIC FANTASY OR HARSH REALITY?

Period: October 1, 1997 - January 31, 2001

Co-ordinator: UNIVERSITY OF CAMERINO, DEPT. OF MOLECULAR, CELL & ANIMAL BIOLOGY Camerino, Italy (F. ESPOSITO)

Objectives

To investigate the impact of insecticide impregnated curtains (IIC) on child mortality over the period 3 to 5 years following their introduction.

Specific objectives:

a) determining whether, after an initial reduction in child mortality following the introduction of IIC, there is a subsequent increase in child mortality ("delayed" mortality) in the age range 6-59 months;
b) documenting changes over the medium term in the ability of children's immune systems to inhibit the invasion of red blood cells by malaria parasites;
c) documenting changes in the prevalence of malaria episodes and in the profile of parasitological parameters of malaria infection in children aged 6-59 months living in villages protected by IIC;
d) documenting changes in the intensity of transmission, in the biting/resting behaviour of malaria vectors, in their susceptibility to permethrin and in the genetic composition of the population following the introduction of IIC;
e) training one Burkinabe graduate to the level of MSc (epidemiology/statistics), one to PhD level in entomology, one to PhD level in immunology, and one to MSc level in molecular biology.

Activities

Child mortality. The primary outcome measure in this study will be all-cause child mortality. This variable will be measured by yearly re-census. At this moment in time the following mortality data are available:

Time period	*Treatment group A*	*Treatment Group B*
1993-94	Mortality in the absence IIC (45.4‰)	Mortality in the absence of IIC (44.0‰)
1994-95	Mortality during 1st year of IIC (27.5‰)	Mortality in the absence of IIC (37.3‰)
1995-96	Mortality during 2nd year of IIC (38.0‰)	Mortality in the absence of IIC (39.9‰)

Complementary activities concern:

Children's immune status. As no marker of protective immunity has been identified thus far, we shall measure *in vitro* the ability of children's sera to inhibit parasite invasion of erythrocytes in combination with a monocyte cell line.

Child morbidity. Cross-sectional surveys in 48 villages (24 in each group) will be conducted in September 1996, 1997 and 1998 (September is the month of peak transmission).

Assessment of transmission. Transmission throughout the study area will be assessed by cross-sectional, outdoor CDC light trap catches. The genetic composition of the vector

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population will be compared between villages of Group A and B and villages outside the study area. The duration of malaria transmission will be assessed longitudinally from June to December. The residual vector population and its biting behaviour will be determined by outdoor CDC light trap catches. Antibodies to the circumsporozoite protein (CS) of *Plasmodium falciparum* will provide an indication of intensity of transmission.

Monitoring coverage and utilisation. Re-impregnation and installation rates are recorded yearly at the time of re-impregnation. Coverage will be further assessed by visits to a 10% random sample of dwellings. Utilisation will be assessed by pyrethrum spray catches and by surprise visits.

Assessment of insecticide susceptibility. Mosquito susceptibility to permethrin will be assessed once yearly, by the standard WHO method on impregnated paper.

Expected outcome

The proposed study will maintain the intervention in both groups A and B (yearly re-impregnation of curtains and replacement of damaged curtains) and the collection of mortality data for an additional three years (up to June 1999). This will provide the following additional data:

Time period	*Treatment group A*	*Treatment Group B*
1996-97	Mortality during third year of IIC	Mortality during first year of IIC
1997-98	Mortality during fourth year of IIC	Mortality during second year of IIC
1998-99	Mortality during fifth year of IIC	Mortality during third year of IIC

It is expected that the opportunity of investing on impregnated materials for controlling malaria in high transmission settings will be evaluable on the basis of the results. In addition, the relationship between transmission, immunity and disease will be clarified.

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Presentation of EC supported joint research projects (1991-1996) continued
STD3
INCO-DC: 1st and 2nd Call

Areas of interest:

2. Schistosomiasis

Contract number TS3*CT910030

DEVELOPMENT OF A VACCINE STRATEGY AGAINST HUMAN AND BOVINE SCHISTOSOMIASIS

Period: April 1, 1992 - March 31, 1995

Co-ordinator: INSTITUT PASTEUR, CENTRE D'IMMUNOLOGIE ET DE BIOLOGIE PARASITAIRE, Lille, France (A. CAPRON)

Objectives

- To optimize the formulation of a recombinant vaccine based on *Schistosoma mansoni* glutathione-S-transferase (Sm28GST) for use preferably in oral immunization against schistosomiasis.
- The vaccine will be used in Phase Ia human trials in Europe.
- As part of a research and control programme in Richard Toll, Senegal, a focus of epidemic schistosomiasis mansoni, to study the immune status of the population before and after chemotherapy.
- In phase Ib clinical trials, non-infected individuals in an endemic area will be immunized.
- Parallel studies will develop a vaccination strategy for the prevention and control of *S. bovis* infection in cattle.

Activities

* The immunogenicity of synthetic peptides and live vectors containing molecular constructs of Sm28GST will be tested using appropriate adjuvants, microencapsulation and oral immunization. This work will be supported with protein crystallography data and 3D-structural analysis of Sm28GST.
* Pre-phase I clinical studies will include measurements of IgE and IgA antibodies and T-cell responses to Sm28GST as well as to *S. mansoni* antigenic extracts.
* Immune and non-immune populations will be identified by studies of reinfection after chemotherapy.
* Phase Ia clinical trials on European volunteers, and Phase Ib trials on non-infected individuals in an endemic country will be planned. In both trials the antibody isotype titers and cytotoxic capacity, T-cell and interleukin responses to Sm28GST will all be measured.
* Calves and goats will be vaccinated with recombinant *S. bovis* 28GST. Measurement of vaccine efficacy will be done by determining both worm and egg loads following experimental challenge with *S. bovis*.

Contract number TS3*CT910030

Expected outcome

⇒ The production of a suitable (mucosal) vaccine formulation based on Sm28GST.

⇒ Phase Ia clinical trials of the vaccine and, depending on success, phase Ib trials.

⇒ Detailed plans for phase II and III clinical trials.

⇒ Evaluation of the immune responses in the epidemic focus in Richard Toll.

⇒ Detailed information on the efficacy of *S. bovis* 28GST as a veterinary vaccine.

Results

Molecular characterization of protective antigens

Several significant advances have been made:

⇒ The crystallization of Sm28GST has been performed and a 3D structure based on X-ray diffusion data and molecular modeling has been obtained.

⇒ The 28GST of *S. haematobium* and *S. bovis* have been successfully cloned. Of particular interest is the fact that comparison of the peptide sequences of *S. mansoni*, *S. haematobium* and *S. bovis* 28GSTs shows interspecific variability in the major protective epitope 115-131, contrasting with a relative conservation of the C-terminal 190-211 epitope.

⇒ The chromosomal gene of Sm28GST has been fully sequenced. The promoter region shows consensus binding sequences for both NF-Y and AP1 transcription factors.

⇒ Several additional genes from *S. mansoni* have been identified and cloned including glutathione peroxidase, superoxide dismutase and calreticulin. The latter is of particular interest due to its preferential localization in reproductive organs (testes, ovary and vitelline glands).

Effector mechanisms and vaccine optimization

⇒ Protective immunity can be induced by a single dose of rSm28GST in rats and this can be related to the production of specific IgE and IgA antibodies involved in the eosinophil-mediated killing of schistosomula. We had previously shown that peptides corresponding to the N- and C-terminal domains of the enzyme could induce the same anti-fecundity effects after vaccination as the entire molecule. Moreover IgA monoclonal antibodies directed against the C-terminal domain were able to mediate the same effects on egg-laying and egg viability.

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⇒ In order to optimize the protective response induced by Sm28GST or its peptides, different strategies have been employed. We have shown that lipopeptide constructs of the C-terminal peptide were much more effective in inducing an anti-fecundity response. In parallel we have explored the ability of the filamentous hemagglutinin (FHA) of *Bordetella pertussis* to induce a strong IgA response after mucosal administration by incorporating the Sm28GST gene in frame with the FHA structural gene. Recombinant *B. pertussis* express the fusion protein on their surface and induce an immune response after nasal administration that protects mice partially against a challenge infection. Reductions in worm burden and fecundity have also been obtained after oral administration of liposomes containing the entire recombinant Sm28GST.

⇒ We have applied the 28GST-based vaccination strategy in outbred animal infection models; ruminants infected by *S. bovis* and *Erythrocebus patas* monkeys infected by *S. haematobium*. A significant reduction in female worm fecundity is consistently induced by vaccination in these models, with notably an 80% reduction in overall egg output in the faeces and urine of Patas monkeys cross-immunized with Sm28GST, a reduction that was sustained over a 48 wk period.

Immune responses to Sm28GST in human populations

⇒ This part of the work was carried out in collaboration with A.E. Butterworth and colleagues in Kenya and more recently in collaboration with B. Gryseels, F. Stelma and our Senegalese colleagues in Richard Toll.

⇒ IgA antibodies to Sm28GST have been shown to be closely associated with the age-dependent acquisition of resistance to infection. Functional analyses revealed that these antibodies inhibited the activity of the enzyme and could impart schistosome fecundity *in vitro*. Current studies in Senegal, although preliminary and limited to pretreatment cohorts, show a relationship between the age-associated decrease in egg output and the IgA response specifically directed against the Sm28GST C-terminal peptide.

Contract number TS3*CT910030

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Contract number TS3*CT920050

EVALUATION OF DEFINED ANTIGEN VACCINES AGAINST *SCHISTOSOMA BOVIS* AND *S. JAPONICUM* IN BOVINES

Period: November 1, 1992 -I April 30, 1996

Co-ordinator: LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE,
DEPT OF MEDICAL PARASITOLOGY,
London, United Kingdom (M. TAYLOR)

Objectives

To evaluate the immunogenicity and protective efficacy of existing and newly defined *Schistosoma bovis* and *Schistosoma japonicum* antigen vaccines in their natural bovine hosts, thereby contributing to the development of a defined antigen vaccine against schistosomiasis.

Activities

* The immunogenicity and protective efficacy of glutathione *S-transferase* (GST), keyhole limpet haemocyanin (KLH) and freeze-thaw schistosomula antigen plus BCG will be assessed in calves.
* *S. bovis* paramyosin will be cloned and evaluated in the same way.
* Native and recombinant GST of *S. japonicum* and KLH will be tested for immunogenicity and protective efficacy in rodents and calves.
* The contribution of serological responses to the immunity to *S. japonicum* stimulated by irradiated cryopreserved, or freeze-thaw schistolomula, will be investigated by passive transfer experiments in mice and calves.
* Antigens recognised by sera of immune bovines from China will be identified and compared with those recognised by rodents immunised with irradiated cercariae.
* *S. japonicum* cDNA libraries will be expressed and purified and recombinant molecules used in crystallographic studies.
* Paramyosin and the Sm23 homologue from Chinese *S. japonicum* will be cloned and tested in rodents and calves.
* *S. bovis* and *S. japonicum* GSTs will be expressed and purified and recombinant molecules used in crystallographic studies.
* IgA monoclonal antibodies to *S. japonicum* GST will be produced and their effects on fecundity and egg viability reported.

Expected outcome

The production of additional defined antigen vaccine candidates.

Results

This work aimed to contribute to the development of defined antigen vaccines for schistosomiasis by evaluating the immunogenicity and protective efficacy of *S. bovis* and Chinese strain *S. japonicum* antigens produced in UK and France in their natural ruminant hosts in Africa and in China. The defined antigens selected for testing included: glutathione *S. transferases*; KLH which bears epitopes cross-reactive with schistosome antigenes; Sj23, the homologue of the protective *S. mansoni* membrane antigen Sm23 and *S. japonicum* paramyosin. 20 full length clones of a 28kDa isoenzyme of *S. japonicum* (Sj28GST) were produced and expressed ready for testing in livestock alongside Sj26GST of a Philippine strain of *S. japonicum* expressed by the pGBEX vector (we showed that this is identical to Sj26GST of our Chinese strain). We likewise cloned and expressed a 3 FE fragment of Chinese *S. japonicum* paramyosin and the large hydrophilic domain of the Sj23 antigen of Chinese strain *S. japonicum*. Further trials were carried out with biochemically-purified native GSTs of Chinese *S. japonicum*, and some trials included KLH, obtained through commercial suppliers. Protection experiments were also carried out using the F/T vaccine (F/T schistosomula of Chinese *S. japonicum* administered with BCG). In the *S. bovis* trials, we used a recombinant version of the 28kDa GST isoenzyme (Sb28GST) together with an

S. bovis F/T vaccine (*S. bovis* F/T schistosomula + BCG) and KLH. Some preliminary immunisation experiments using *S. japonicum* antigens were also carried out in BALB/c mice to explore the usefulness of the mouse model in this type of vaccine development programme.

The most important finding was that each of our panel of *S. japonicum* recombinant derived antigens induced significant levels of protection in sheep. Further work is now needed to optimise vaccination schedules, and to devise the most effective vaccine delivery systems for safe and effective immunisation of farm animals, and ultimately, humans.

The *S. bovis* trial in cattle in Sudan demonstrated that r-Sb28GST had some protective efficacy, though it was less effective than vaccination with unfractionated native SbGSTs which had been tested previously, perhaps indicating that other SbGST isoenzymes are also required for the induction of high-level resistance in this model. The *S. bovis* F/T vaccine, modelled on the *S. japonicum* F/T vaccine also showed some protective effects, but these were far less dramatic than we obtained in the *S. japonicum* work. In the cattle/*S. bovis* system, protection was manifested entirely as a reduction in worm fecundity, rather than a reduction in adult worm recovery.

Protection was obtained in BALB/c mice vaccinated with rSj23, but little evidence of protection was obtained using native or recombinant paramyosin in BALB/c mice. Thus although the mouse model may well be of value for investigating the immunogenicity of *S. japonicum* candidate vaccine molecules, it is probably preferable to carry out the protection experiments in the natural ruminant hosts.

In an attempt to enhance vaccine-induced protection, co-administration of two different antigens was attempted for both *S. bovis* in cattle (using rSb28GST and KLH) and *S. japonicum* in sheep and water buffalo (using native SjGST and KLH). No evidence of significant additional protection was obtained in these co-administration experiments.

Finally, screening of our Chinese *S. japonicum* strain libraries with immune sera led to the identification and characterisation of two novel antigens of vaccine potential, calreticulin and calpain, which we plan to evaluate in large animal protection experiments.

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Contract number TS3*CT920118

CELL MEDIATED IMMUNITY TO SCHISTOSOMES. EVALUATION OF MECHANISMS OPERATING AGAINST LUNG STAGE PARASITES, WHICH MIGHT BE EXPLOITED IN A VACCINE

Period: December 1, 1992 - November 30, 1993

Co-ordinator: UNIVERSITY OF YORK, DEPT. OF BIOLOGY
York, United Kingdom (R. WILSON)

Objectives

To evaluate cell mediated immune responses to lung stage parasites of *Schistosoma mansoni* in mice and in infected humans.

Activities

* Secreted and soluble antigens derived from schistosomula, cultured *in vitro* for eight days, will be used to expand T-cell clones and lines generated from lymph node populations recovered from mice shortly after intradermal vaccination with day 8 attenuated schistosomula.

* Of the phenotypic and functional assays to be employed, proliferation and interferon gamma production by clones or lines when co-cultured with live lung-stage schistosomula are considered most important. Clones which meet these criteria will be tested *in vivo* for the ability to mediate delayed type hypersensitivity responses and reduce maturation of parasite infections relative to irrelevant T-cell clones.

* Following clinical and epidemiological investigations, including assessment of resistance to reinfection after chemotherapy, lymphocytes will be collected from the peripheral blood of patients from Bela Fama and the profile of their cytokine production (IL2, IL3, IL4, IL5 and Interferon gamma) in response to the same larval antigens will be described.

Expected outcome

⇒ The establishment of T-cell clones which meet the criteria of proliferation and interferon-gamma production, and the measurement of human cytokine profiles in response to lung stage antigens.

⇒ Development of a longer-term project.

Contract number TS3*CT920118

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Contract number TS3*CT940296

GENETIC AND IMMUNOLOGICAL FACTORS IN HUMAN RESISTANCE TO *SCHISTOSOMA MANSONI*

Period: September 1, 1994 - August 31, 1997

Co-ordinator: INSERM U399, FACULTE DE MEDECINE, DEPT. IMMUNOLOGY AND GENETIC OF PARASITE DISEASES,Marseille, France (A. DESSEIN)

Objectives

- To determine to which extent genetic factors control susceptibility to infection and to disease in subjects living in an endemic area (Brazil) and in subjects who recently migrated from a non-endemic area of *S. mansoni* (Kenya).
- To identify and characterize the mode of action of susceptibility genes.
- To evaluate two schistosome antigens as vaccine candidates.
- To develop a strong group of immunology at the Faculty of Medicine of Uberaba.
- To strength the expertise of the group of Salvador in the field of schistosomiasis.

Activities

* Epidemiological studies to evaluate the weight of environment and behaviours on infection and morbidity.
* Search by segregation analysis of gene(s) with a major effect on infection and morbidity.
* Mapping of these genes by linkage analysis using the microsatellite method.
* Analysis of the anti-parasite immune response of susceptible and resistant subjects to uncover an immunological deficit in genetically susceptible individuals.
* Analysis of the lymphokine production pattern of T-lymphocytes in subjects with various degree of fibrosis.
* Purification, cloning and production of the active antigen in F28 fraction. Mapping of antigenic determinants of Sm37.
* Evaluation of the cellular and antibody response of resistant and susceptible subjects to recombinant protein and to peptides.

Expected outcome

⇒ The demonstration that genetic predisposition to infection and disease accounts, to a large extent, for the heterogeneous distribution of clinical phenotypes in endemic area of *Schistosoma mansoni.*
⇒ The demonstration that such a genetic predisposition results from the action of a few genes (major genes) controlling infection and regulating Symmer's fibrosis.
⇒ The identification of immune mechanisms that play a critical role in human defences against *S. mansoni.*

Progress toward the vaccine through the identification of:
⇒ The protective immune mechanisms to be stimulated by the vaccine;
⇒ The immunological deficit that must be "overcome" by the vaccine in genetically susceptible subjects;
⇒ Vaccinating antigens.

Strengthening of research capabilities of two brazilian laboratories

This project is based on a long standing collaboration between our groups; it is grounded on observations made by us in Brazil on a major gene in human resistance to *S. mansoni* and on the immunological mechanisms that are critical to human resistance to infection.

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A similar immunological approach has been taken in Kenya, interactions between the partners will allow the determination of whether the observations on the genetic control of resistance can be extended to another endemic area. This project has a major training component for young scientists.

Results

⇒ Evidence has been obtained by segregation analysis for the existence of a major gene or a major locus in the control of infection intensities.
⇒ This major gene (Sm 1) has been located on chromosome 5q31-33.
⇒ Analyses of the immune response of homozygote resistant and susceptible subjects have demonstrated differences in lymphokine production. Resistance is associated with a Th0/2 type lymphokine production and susceptibility is associated with a Th0/1 type of lymphokine production pattern.
⇒ The active molecule in F-28 has been purified and cloned. It is referred to as Sm10. Antigenic determinants have been mapped on Sm37.
⇒ Cellular and antibody response to these antigens are being evaluated in resistant and susceptible subjects.

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CELL MEDIATED IMMUNITY TO SCHISTOSOMES: EVALUATION OF MECHANISMS OPERATING AGAINST LUNG STAGE PARASITES, WHICH MIGHT BE EXPLOITED IN A VACCINE

Period: October 1, 1994 - September 30, 1997

Co-ordinator: UNIVERSITY OF YORK, DEPT. OF BIOLOGY
York, United Kingdom (R. WILSON)

Objectives

- To identify and clone the antigens mediating lung phase immunity to *Schistosoma mansoni* in mice vaccinated with irradiated cercariae.
- To use the recombinant antigens for evaluation of T cell responses in mice exposed to the irradiated vaccine, and for protection experiments.
- To examine the responses of peripheral blood lymphocytes from human patients with different clinical forms of schistosomiasis, to recombinant lung stage-antigens.
- To analyze selected lung stage antigens at the molecular level by mapping B and T cell epitopes using synthetic peptide constructs.

Activities and Results

Molecular Biology of Schistosome Antigens

Experimental work performed during this project has sought to use several different techniques to pinpoint potential vaccine antigens. This has involved the identification of cDNAs encoding such molecules, their sequence analysis, and the subsequent production and purification of recombinant proteins in *E. coli*. A secondary aim has been to identify and subsequently characterize genes expressed uniquely by lung-stage schistosomula using the technique of RAP-PCR. We have also investigated the possibility of identifying cDNAs encoding putative transmembrane or secretory proteins from expressed sequence tags produced as part of the schistosome genome project.

Serum raised in rabbits against proteins released by lung-stage schistosomula during *in vitro* culture has been used to screen both lung-stage and adult worm DNA libraries. A novel clone coding for a protein of Mr 16.4kDa (A26) has been identified, sequenced, expressed in a pET vector, and purified. A monospecific serum has been produced against the recombinant A26 protein and used to identify the "native" parasite protein by probing Western blots of soluble preparations of cercariae (SCAP), lung-stage schistosomula (SLAP) and adult worms (SWAP). Preliminary results indicate that a protein of approximately 31kDa is detected in all three preparations, but appears to be most abundant in cercariae. The same screening procedure also identified three previously described vaccine candidates, paramyosin, myosin and calpain, the last of which we are pursuing further.

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In addition to the clones identified by screening lung-stage and adult worm DNA libraries with sera raised against lung-stage proteins, secretory proteins from other life-cycle stages have been sought. A schistosome calcium-binding protein and a 21.7kDa antigen have been identified from a cercaria DNA library, plus a putative cytochrome C and four unknown clones. Screening of an adult worm library with antibodies directed against released proteins resulted in the isolation of another four unknown clones, plus previously sequenced *S. mansoni* HSP70, cathepsin and a known, secretory protein, LGG. A number of the unknown clones have been sequenced more extensively and subsequently expressed in the pQE or pET vectors. As well as being vaccine candidates, such proteins may have potential use as markers of disease progression in human patients, and will enable the immunogenicity of proteins released at different life-cycle stages to be compared in various assays.

The contribution of the Lille Pasteur Institute group to the project has involved the subcloning and expression of cDNAs encoding some of the proteins potentially involved in the protective immune response directed against lung schistosomula of *S. mansoni*, namely calpain, the tegumental antigens Sm22.6 and Sm21.7, and a DNA encoding an *S. mansoni* homologue of mammalian epididymal secretory protein I. Attempts to express these four cDNAs are currently underway in Lille but significant difficulties have been experienced with all of the clones. A variety of vectors are therefore being tried to circumvent the problems.

In a new departure, the lung stage library constructed in York has been transferred to our partners in Belo Horizonte for screening with sera from patients in the acute phase of the disease and five clones (HL-1 to HL-5) were obtained. All five have been sequenced and expressed in either pET or pQE vectors and are now at the purification stage.

Assessment of immunogenicity

Now that we have developed a panel of recombinant proteins representative of molecules released by parasites at various stages throughout the life-cycle, the immunogenicity of each can be assessed. Since parasite-specific Th1 cells play a pivotal role in the effector response in once-vaccinated mice, most attention will focus on assays of T cell proliferation and cytokine production. The secretion of cytokines by cells recovered from the lungs of mice 17 days post-vaccination will be our main indicator of protein immunogenicity since it is this sub-set which is responsible for challenge parasite elimination. In particular, we shall be seeking proteins which stimulate high levels of IFNγ production. We are also developing an *in vivo* assay of antigen reactivity, by injecting recombinant proteins into the pinnae of mice previously exposed to the irradiated vaccine to measure delayed-type hypersensitivity responses.

In collaboration with our Brasilian partners, we are currently testing the ability of peripheral blood mononuclear cells (PBMC) from patients with different clinical forms of schistosomiasis to proliferate *in vitro* in response to each recombinant protein.

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Four recombinant antigens have been tested so far; all elicited responses above background but considerably lower than those to SWAP and SEA preparations (this is not unusual with recombinants and represents an obstacle to their evaluation as vaccine candidates by direct probing of human responses). In an alternative screen, we are using an ELISA to determine total IgG responses to each recombinant of patients in the various clinical categories of schistosomiasis, as an indicator of preceding T helper cell responses.

Human schistosomiasis in Brazil

Field surveys of schistosomiasis in the vicinity of Belo Horizonte have continued, concentrating on the district of Sabara. Stool sampling of 1413 residents revealed an overall prevalence of 27%, with a mean egg count of 58 epg (range 5 - 633). Of these infected individuals, only 12% presented egg counts above 500 epg which might be considered a heavy infection; few showed evidence of advanced hepato-splenic disease. For this reason, we have continued our studies of human responses to *S. mansoni* in an endemic area of Northern Minas Gerais state, centered on Corrego Bernardo, using a field laboratory in the city of Governador Valadares.

Both cellular and humoral responses to SLAP, SWAP, and SEA have been evaluated. *In vitro* stimulation of PBMC with SLAP leads to a significant proliferative response in patients with the different clinical forms of the schistosomiasis, except in hepatosplenic individuals. Neutralisation of cytokines in PBMC cultures reveals that the proliferative response to SLAP is differentially regulated from that to SEA and SWA, with no effect observed following the addition of antibodies to IL-4, IL-5 and IL-10. On the other hand, addition of anti-IFNγ antibodies to the PBMC cultures readily decreased the proliferative response to SLAP as it did for SWAP and SEA.

Special emphasis has been given to the development of methods for the intracytoplasmic staining for cytokines that would allow for their simultaneous identification together with the cell type secreting them, using flow cytometry. Initial intracytoplasmic staining data was obtained for IL-2, IL-4, IL-5 and IFNγ in PBMC. Patients were divided into IgE^{hi} and IgE^{lo} groups on the basis of their serum responses to SWAP. The results demonstrate that the IgE^{hi} group had a significantly higher frequency of IL-4 and IL-5 positive cells than the IgE^{lo} group. The frequency of IFNγ positive cells was the same in both groups but, whilst in the IgE^{hi} group the ratio for IFNγ/IL-4 was 13, in the IgE^{lo}group it was 35. Taken together, these results correlate IL-4 directly and IFNγ inversely with serum IgE levels. It is important to note that in this study all patients were age-matched, thus no influence of age can be attributed to the observed results. Furthermore, all individuals were from the same area and to date, the differences in the two groups of patients cannot be attributed to different levels of water contact.

We have also demonstrated that exposed, "normal endemic" individuals have a high IgE antibody response which is elevated only to schistosomula tegumental antigens. We have previously demonstrated that PBMC from "normal endemic" individuals secrete significant levels of IFNγ when stimulated *in vitro* with SWAP or SEA.

Contract number TS3*CT940303

The data obtained with the intracytoplasmic staining for cytokines demonstrated that infected patients with low levels of anti-SWAP IgE have the highest frequency of INFg positive cells. We have postulated that the effective immune response to a *S. mansoni* infection is multifactorial and that it is site-dependent, i.e. different compartments may develop distinct effector responses to the invading parasite. In this context, it is possible that the early attrition may be mediated by IgE antibodies and the later, as the parasite migrates through the lungs, the immune effector mechanism becomes mainly IFNγ-dependent. The correlation between the intracytoplasmic staining of cytokines and development of resistance to infection, or pathology is not yet adequate to determine whether there is a cause/effect relationship and whether one can distinguish between the roles of Th1 and Th2 responses in these situations.

One of the major criticisms of work on human immune responses to *S. mansoni* infection has been that the studies are always performed using PBMC, which may not reflect the events in the lymphoid organs. To address this question we have compared the phenotype of cells present in the peripheral blood and in the spleens of hepatosplenic patients. We observed an increase in $CD4^*HLADR^+$, $CD5^+CD19^+$, $CD8^+HLADR^+$ and NK cells in both compartments, relative to that in normal non-infected/non-exposed controls (accident victims). These results demonstrate that analysis of the peripheral blood reflects the findings in a lymphoid organ, such as the spleen.

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Contract number IC18*CT970212

EVALUATION OF THE REDIATION-ATTENUATED SCHISTOSOME VACCINE IN PRIMATES AS A MODEL FOR HUMAN VACCINE DEVELOPMENT

Period: January 1, 1998 - June 30, 2000

Co-ordinator: UNIVERSITY OF YORK, DEPARTMENT OF BIOLOGY
York, United Kingdom (A. WILSON)

Objectives

- To investigate the radiation-attenuated (RA) schistosome vaccine in chimpanzees and simultaneously examine the development of the granulomatous responses to schistosome egg disposition in the liver of control animals.
- To compare the immunological and pathological responses of human patients, exposed to schistosome infection in endemic areas of Brazil, with those of the chimpanzees.
- To explore in baboons aspects of the RA vaccine, crucial to its evaluation as a model for a human recombinant vaccine.

Activities

A core vaccination experiment will be performed involving three test and three control chimpanzees.

The immune responses to vaccination and challenge will be compared in two distinct physiological compartments, the peripheral blood and the airways of the lung. Serum will also be obtained for determination of specific antibody responses. Leucocytes will be recovered from blood for determination of antigen-driven proliferation and cytokine production after 72-96h of *in vitro* culture. Lymphocytes in whole blood will be phenotyped by flow cytometric analysis. Airway leucocytes will be recovered from test and control animals by bronchoalveolar lavage over the vaccination period to determine whether the lungs have been pre-armed with schistosome-reactive cells. The efficacy of vaccination after challenge with normal cercariae will be estimated from faecal egg counts. Mature worm burdens will also be estimated by the measurement of parasite gut-derived circulating antigens (CAA and CCA) in serum and urine. The pathogenic mechanisms operating after the start of egg deposition in challenged animals will also be intensively monitored. The liver will be sampled at regular intervals by needle biopsy. A wedge surgical biopsy will be taken late in the study. The recovered tissue will be subjected to a detailed histopathological and immunocytochemical analysis. Observations will be made on the gross pathology induced by a schistosome infection by estimating the extent of hepatic fibrosis using non-invasive ultrasound scanning of the liver.

Human responses to schistosome infection will be evaluated in a cross-sectional study of Brazilians patients during the acute and chronic phases of the disease. Characterization will use primarily peripheral blood leucocytes, but also cells from other compartments that may become available. Phenotypic analysis of lymphocytes will be performed for the same range of CD markers as in chimpanzees. The expanded T cell populations will also be phenotype to pinpoint the characteristics of schistosome-reactive T cell subsets. Cytokine levels in all PBL cultures will be evaluated by ELISA. Biopsy samples of livers and spleen, obtained from patients with chronic (hepatosplenic) disease, will be analysed by immunocytochemistry.

Two experiments will be undertaken in baboons to define further certain parameters of the RA vaccine. The question of whether protection is long-lasting will be addressed in an experiment requiring 15 test and 15 control baboons. The test animals will be given 3 vaccinations using the same pools of parasites. Protection will then be measured by portal perfusion to determine adult worm burden at one, three and six months by challenging five test and five control animals on each occasion.

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A second experiment is designed to discover whether there is a celling to protection. Three groups of test animals will receive five, three of one vaccinations, respectively, before they and a single group of controls are all challenged with the same pool of normal cercariae; protection will be measured six weeks after challenge. Assays of immune reactivity, including antigen-driven proliferation, secretion of some cytokine proteins (IL-2, 4, 5 and IFNγ), and specific IgG titres, will be performed. Serum samples will be obtained at perfusion, to determine circulating antigen levels.

Expected outcome

Immunological parameters will be monitored in primates from the start of experiments so that the effects of exposure to the RA vaccine should rapidly become apparent. In the case of chimpanzees, the most crucial sampling period will be after challenge with normal cercariae when the level of protection achieved will be determined by indirect methods. In the case of the baboon experiments, a definitive estimate of protection will be obtained by counting the portal worm burdens. In both situations, the level of protection will be related to the range of immunological parameters measured. Studies in human patients in Brazil will take place in parallel with the primate vaccination experiments and attempts will be made to relate these to the experimental findings in order to evaluate the utility of the primates as models for human schistosomiasis. These cross-comparisons will also be important in the studies on liver pathogenesis in the chimpanzee.

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Contract number IC18*CT970240

EFFICACY OF rSh28GST AS AN ANTIFECUNDITY VACCINE AGAINST *SCHISTOSOMA HAEMATOBIUM* INFECTION (II): IMMUNE MECHANISMS IN EXPERIMENTAL MODELS AND IN MAN

Period: October 1, 1997 - September 30, 1999

Co-ordinator: INSTITUT PASTEUR, DEPT. OF IMMUNOLOGY & PARASITOLOTY, Lille, France (A. CAPRON / G. RIVEAU)

Objectives

To evaluate a well-founded vaccine strategy against the trematode parasite *Schistosoma haematobium* by evaluating:

- the immune responses towards, the 28 kDa glutathione *S*-transferase (Sh28GST) and its major epitopes, in infected human populations in three endemic areas;
- the present epidemiological status and transmission process in each endemic area;
- the feasibility of the use of new mucosal delivery systems for the recombinant subunit vaccine.

In addition, the project will promote the development of south-south collaborative research between the participating DC partners, in terms of technological and staff exchanges. The bi-directional programme (North-south) for research training which took place in 1995 for the cellular immunity analysis and epidemiological and medical studies, will be continued.

Activities

* Epidemiological survey of *S. haematobium* infection in Senegal, Niger and Madagascar. Study of the transmission process of the disease.
* Immuno-epidemiology of *S. haematobium* infection in human before and after treatment. Humoral and cellular responses to rSh28GST in infected human populations, in Senegal, Niger, and Madagascar.
* Production of rSh28GST, liposomes and nucleic vaccine vectors. Humoral responses to mucosal immunization in mice and Patas monkeys,
* Mouse model of *S. haematobium* infection (parasite life cycle). Humoral and cellular responses in infected mice, mucosal vaccination trials in mice.
* Monkey model of *S. haematobium* infection. Humoral and cellular responses in infected animals, mucosal vaccination trials.
* Training of one Senegalese technician in immunochemistry, one physician scientist in epidemiology from Niger, and one scientist in Madagascar for cellular immunology will be undertaken. Training of two European students in human immunology, one for the studies in Madagascar (March 1998), the other one in Senegal (June 1998).

The coordination of external services for toxicology analysis, and Phase I a clinical trial of rSh28GST in Europe will be engaged by Partner 1.

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Expected outcome

The epidemiology and immuno-epidemiological studies on *S. haematobium* infected populations will give us an up to date view of the parasitic status and the specific immune status of the targeted populations allowing us to prepare the appropriate schedule for vaccine trials in infected populations (phase 2; Sh28GST in Alum), which is planned in 1999.

The studies on the proposed new vaccine formulations for mucosal immunisation will be the last step of their evaluation. Their efficacy will be confirmed, and their capacity under field conditions will be evaluated.

The project will ensure the training of DC partner technicians, doctoral and post-doctoral students and technology transfers, notably between the DC partners. We place particular emphasis on the development of this collaboration in the context of the regional integration of research efforts against infectious diseases.

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Contract number IC18*CT970243

DEVELOPMENT OF A VACCINE FOR SCHISTOSOMIASIS JAPONICA

Period: October 1, 1997 - September 30, 2000

Co-ordinator: LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE,
DEPT. OF INFECTIOUS AND TROPICAL DISEASES,
London, United Kingdom (M.G. TAYLOR)

Objectives

To develop a vaccine for schistosomiasis japonica.

Activities

We will evaluate the protective efficacy of novel formulations of several recombinant *S. japonicum* antigens in their natural sheep and pig hosts. We will develop improved methods of vaccine delivery by preparing and testing antigens delivered as DNA vaccines or in liposomes. Also, with a view to planning eventual clinical trials with one or more of these antigens, evidence of a protective role for antibody or cytokine responses to each of these antigens will be investigated using sera and whole-blood assays from "resistant" and "susceptible" *S. japonicum* patients in China. Antigens to be developed are those which we have already cloned, expressed, and tested in animal vaccination experiments: glutathione S-transferases, paramyosin, myosin, a 23kDa integral membrane protein, and Sj22.6.

Expected outcome

Cloning work for production of recombinant paramyosin, Sj23, Sj22.6, and myosin will be completed and DNA vaccine constructs of Sj23 and Sj28GST will be produced. These preparations will be scaled up and tested for immunogenicity and protective efficacy/studies of immune mechanisms in mice. Liposome formulations of paramyosin, Sj28GST, Sj23, myosin and Sj22.6 will be produced and tested for immunogenicity and protective efficacy in mice. Promising formulations will be further tested in sheep and pigs, and the antigenicity of the recombinants will be determined in humans.

This work programme will establish the optimal formulations of our panel of recombinant *S. japonicum* antigens for subsequent field testing in livestock. The human studies should give indications as to which if any of these antigens is associated with immune protection against reinfection in man. Ultimately this work may lead to the introduction of effective vaccines for use in both humans and their domestic animals.

Contract number IC18*CT970243

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Contract number TS3*CT910040

QUANTITATIVE DIAGNOSIS OF SCHISTOSOMA INFECTIONS BY MEASUREMENT OF CIRCULATING ANTIGENS IN SERUM AND URINE

Period: March 1, 1992 - February 28, 1995

Co-ordinator: RIJKSUNIVERSITEIT LEIDEN, DEPT. OF PARASITOLOGY
Leiden, The Netherlands (A.M. DEELDER)

Objectives

- The development and optimization of enzyme-linked immunosorbent assays (ELISA) and reagent strips for the quantitative detection of circulating anodic (CAA) and circulating cathodic (CCA) antigens and other adult worm antigens (CA-2), immune complexes, and for the demonstration of several antigens in one assay.
- The evaluation of circulating antigen detection systems for use as sero-epidemiological tools in studies of the chemotherapy, immunology and morbidity of *Schistosoma mansoni*, *S. haematobium*, *S. japonicum* and *S. intercalatum* infection.

Activities

* Monoclonal antibodies directed against schistosome circulating antigens will be used in ELISA assays. Once monoclonal antibodies are produced against circulating antigens of *S. japonicum*, an ELISA assay will be established and optimized for detection of this species.
* Appropriate, alternative, simple procedures for pre-treating urine specimens will be investigated.
* The daily fluctuations of antigen concentration in urine and serum will be investigated.
* Reagent strip ("dipstick") assays will be employed in an attempt to develop a simple, field applicable assay.
* Following training and technology transfer, sero-epidemiological surveys and clinical field trials will be conducted.

Expected outcome

⇒ Improvement in the sensitivity and specificity of the circulating anodic (CAA) and circulating cathodic (CCA) antigen detection ELISA assays for *Schistosoma mansoni, S. haematobium* and *S. japonicum*.

⇒ Evaluation of antigen detection assays as sero-epidemiological tools.

⇒ The development of a reagent strip assay.

⇒ The successful transfer of ELISA, reagent strip and immunochemical expertise to the DC partners.

Contract number TS3*CT910040

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Contract number IC18*CT970241

FIELD APPLICABLE ANTIGEN ASSAYS FOR HUMAN SCHISTOSOMIASIS: A MULTI-CENTRE DEVELOPMENT AND IMPLEMENTATION STUDY

Period: October 1, 1997 - September 30, 2000

Co-ordinator: UNIVERSITY OF LEIDEN, Dept. of Parasitology, Leiden, The Netherlands (A.M. DEELDER)

Objectives

- field assays for detection of schistosome antigens CAA and CCA in serum and urine will be developed in reagent strip and/or filter format using colloidal gold-labelled monoclonal antibodies (Mabs).
- assays will be tested in pilot field trials in parallel with magnetic bead assays for detection of CAA and CCA in serum and urine of patients infected with the major *Schistosoma* species (*S. mansoni*, *S. haematobium*, *S. japonicum*).
- selected assays will be applied in sero-epidemiological studies, in chemotherapy follow-up, and reinfection studies (*S.m.*, *S.h.*) for assessment of antigen kinetics (in relation to egg output).
- new MAbs for detection of circulating antigens will be developed for *S. japonicum* in particular, and tested and applied in assays as described above.

Activities

Using McAbs already developed and optimized for ELISA's detecting parasite antigens in serum and/or urine, the format of a lateral flow diagnostic device (cf. pregnancy test) will be evaluated and optimized. This format is based on the reaction of applied sample first passing and dissolving a dried labelled antibody, and subsequently migrating to a zone of bound capture antibody. This technology format is now becoming commercially available and the necessary contacts have already been made. This approach will result in a very rapid assay (approx. 4 minutes) but probably with a sensitivity only high enough to identify moderate and heavy infections.

Starting at the end of the first year, the field-applicable assays developed in Leiden will be evaluated by the three partners in the endemic areas in study groups of about 50 infected and 50 uninfected individuals. For these studies, the rapid ELISA will be used as a reference assay. It is anticipated that for each of the *Schistosoma* species this number of samples and the available reference data will allow selection of the assay formats most appropriate for addressing specific studies in larger study cohorts. As follow-up of chemotherapy will be essential for evaluating sensitivity of assays before and after chemotherapy, such studies will form an integral part of the work on each of the *Schistosoma* species. In all studies, a 5% sample will be randomly selected for quality control of the assays, initially to be performed in Leiden, and at a later stage in the laboratory of one of the African DC Partners (e.g. in Zimbabwe).

The assays thus developed will rapidly be transferred to DC partners for evaluation under field conditions. Technology will also be transferred to the Institute of Parasitic Diseases in Shanghai, China, to allow optimization with the anti-*S.j.* McAbs.

Contract number IC18*CT970241

The hydrophobic, chemically inert polypropylene fibre web material, which was developed by the Dept. of Medical Microbiology and Immunology in Göteborg in collaboration with Mölnlycke AB, Sweden, as sampling material for blood containing CAA, will be further investigated. It is anticipated that the fibre web sampling format could in a later phase of the project be included for quality control studies of samples which have been tested in the field.

Sample pre-treatments, although currently already relatively simple, will be further adapted for field use and applicability to larger sample sizes.

Expected outcome

⇒ a field applicable diagnostic assay for schistosomiasis
⇒ a field applicable sample pre-treatment method
⇒ a field applicable sample collection and storage method based on fibre web
⇒ anti *Schistosoma japonicum* McAb cell lines applicable in specific and sensitive assays

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Contract number TS3*CT910041

EPIDEMIOLOGY, SEROLOGY AND CHEMOTHERAPY OF *SCHISTOSOMA MANSONI* INFECTIONS IN A RECENTLY EXPOSED COMMUNITY NEAR RICHARD TOLL, SENEGAL

Period: March 1, 1992 - February 28,1996

Co-ordinator: PRINCE LEOPOLD INSTITUTE OF TROPICAL MEDICINE
Antwerp, Belgium (B. GRYSEELS)

Objectives

The project initially had four main, inter-related objectives:

- To study patterns of infection and reinfection with *Schistosoma mansoni* and of humoral immune responses in a non-immune, recently exposed community near Richard Toll, Senegal, and to test hypotheses concerning the role of acquired immunity in humans.
- To apply to this end, assays for antigen detection in serum and urines besides classical epidemiological, parasitological and immunological (antibody patterns) methods, and to evaluate these techniques as a monitoring tool for intervention studies and/or as epidemiological research tool.
- To provide baseline data for other immuno-epidemiological and intervention studies, including possible vaccine development; and to contribute to the epidemiological, serological and chemotherapeutic components of these other studies.
- To develop and evaluate optimal chemotherapeutic strategies in order to prevent the development of severe morbidity in this new focus, and to contribute to the development of durable, integrated control measures.

In the course of the project, additional objectives were added as they became obviously important:

- To study the morbidity patterns in this recently but heavily infected population.
- To investigate the apparent and unexpected failure of praziquantel, the recommended drug for schistosomiasis, to cure most infections.

Activities

* Prevalence and intensity of infection were measured in four randomly selected population samples of 400 individuals, at intervals of eight months, in the village of Ndombo, Northern Senegal. Surveys consisted of repeated faecal egg counts, collection of serum and urine, standardised medical history and clinical examination.
* In each cohort, infected subjects were treated with 40 mg/kg praziquantel; acceptability and side-effects were monitored. Parasitology, serology and morbidity were follow-up at 6 and 12 weeks, and at 12 and 24 months after treatment.
* Serological samples were screened for subclass specific antibody responses to crude and defined antigens with immunogenic or immunodiagnostic potential.
* Circulating anodic and circulating cathodic antigens were measured in urine and serum and evaluated against conventional parasitological results.

* During the follow-up, quantitative malacological studies and water contact observations were conducted at five major transmission sites in Ndombo. Reinfection rates were related to exposure to infection and immunological profiles.
* Alternative drug regimens and control measures were evaluated.

Results

Immuno-epidemiological studies: infection and reinfection patterns

The cross-sectional study of the four cohorts showed extremely high prevalences (>90%) and intensities (25-40% excreted more than 100 epg) of infection in this community. Prevalences rapidly increased in children, and from 5 years of age the whole population was infected. The age-dependent intensity curves peaked strongly and consistently in children, to drop in adults, although, on the basis of observations in other foci, acquired immune resistance should not have yet developed in this focus.

The studies of antibody response revealed that IgE levels increased with age, while IgG4 peaked in the age-group 10-19 years. IgE and IgG4 levels against adult worm antigen (AWA) and soluble egg antigen (SEA) increased between cohort 1 and cohort 4 in almost all age-groups. In all cohorts examined a strong correlation between IgG4 and pre-treatment egg load was observed. Further analysis and comparison with chronically infected populations are in progress.

Circulating antigen detection

Circulating antigen detection (CAA and CCA) in urine and serum besides faecal egg counts was used, with as objectives: (1) to apply and evaluate the circulating antigen detection assays as an epidemiological tool; (2) to determine the relationship between worm burden and egg output, by comparing antigens levels with those in endemic areas; and (3) to investigate the use of these antigens assays for the assessment of cure following chemotherapy.

⇒ In the first cohort, the use of antigen detection confirmed the extremely high prevalences and intensities of *S. mansoni* infection as determined by stool examination. Serum CAA provided intriguing epidemiological information on worm burdens, while urine CCA showed promise for non-invasive diagnosis and screening.

⇒ The comparison of the Ndombo situation (recent focus) with a similarly intense, but chronic focus of transmission of *S. mansoni* in Maniema, Eastern Zaire, showed that while parasite egg counts were almost similar in the two populations, serum levels of both CAA and CCA were approximately twice as high in the chronically exposed community.

⇒ The antigen assays confirmed the poor efficacy of praziquantel. No significant differences in cure rates between the two groups treated with two different dose regimens of praziquantel (40 mg/kg in one oral dose, and 30 mg/kg in 2 oral doses at 6 hour-interval) were observed.

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Morbidity

The results of ultrasonographical studies confirmed the surprisingly low frequency of hepatosplenic pathology and the high intensities of infection. There was no correlation between frequency of symptoms and egg counts. Preliminary data showed that early anti-schistosomal treatment reduced early and mild hepatic morbidity despite the fact that it does not prevent reinfection. The study further allowed the optimisation of ultrasound methods and revealed a potential new application of ultrasound for assessing intestinal schistosomiasis pathology. It also raised the need to revise the current grading systems.

Efficacy of treatment with praziquantel

The studies of praziquantel efficacy repeatedly and convincingly confirmed early field observations of low cure rates after praziquantel treatment in this focus, even with the use of a higher dosage (2 x 30 mg/kg). Interestingly, normal (high) cure rates were obtained with oxamniquine, the only available alternative drug. Further collaborative studies are continuing to elucidate the mechanism of drug-resistance.

Outcome

⇒ Increased work interactions, exchanges, training and capacity transfer and strengthening. Several young researchers from Senegal, and Europe, have been and/or are currently training at Msc, MD and PhD levels have actively joined the EC-Schistosomiasis network and are responsible for of new research and control projects in Senegal.

⇒ New insights in the epidemiology of schistosomiasis.

⇒ Confirmation of epidemiological use of circulating antigen detection.

⇒ Development of ultrasound protocols for intestinal morbidity, and standardised, comparative assessment of intestinal and hepato-splenic morbidity in different areas and types of foci.

⇒ A methodological and multidisciplinary approach to praziquantel-resistance in *S. mansoni*

⇒ Scientific bases and capacity building for the development of an operational control programme.

Contract number TS3*CT910041

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Contract number TS3*CT920066

IMMUNITY AND MORBIDITY IN HUMAN *SCHISTOSOMA MANSONI*

Period: October 1, 1992 - September 30, 1995

Co-ordinator: UNIVERSITY OF CAMBRIDGE, DEPT. OF PATHOLOGY
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(A. BUTTERWORTH / D. DUNNE)

Objectives

- To provide a framework for a national schistosomiasis control programme based on previous research on the impact of chemotherapy.
- To analyze potentially protective immune responses, in particular TH2 and TH1 responses and specific anti-schistosome IgE and IgA antibodies.
- To investigate the responses to candidate vaccine antigens, particularly the *Schistosoma mansoni* glutathione-S-transferase (P28), and a 22kDa antigen recognised by IgE from immune individuals.
- To screen *S. mansoni* λGT11 cDNA libraries with human infection serum for new potential vaccine candidate antigens recognised by IgE antibodies.
- To study the interactions between *S. mansoni* infection, nutritional status and other infections, especially malaria, in the development of morbidity.

Activities

* Field work will be carried out near Kambu, Machakos District and Kangundo, Kenya, using an established field work infrastructure which permits detailed and systematic observations on parasitology, malacology, water contact, cercariometry, anthropometry and pathology.
* Studies of potentially protective immune responses will be made in different patient groups:
  — Study H will be done on samples from 140 individuals (age 6-60 years) obtained in 1985-88.
  — Study I comprises 90 individuals (age 10-40 years) on which data on repeated chemotherapy and reinfection (1990-92) are available. Lymphocyte culture supernatants and serum samples from these groups will be analyzed in relation to levels of reinfection.
* Group I will be re-treated yearly between 1992 and 1995 (Study IR), and bled 3 and 10 months after treatment, in order to study a possible boosting effect of chemotherapy on immunological responses. A new cohort of schoolchildren (age 8-9 years) (Study CR) will be treated annually and bled 4 months later from 1992 onwards in order to assess longer term effects of boosting.

* Lymphocytes from studies IR and CR will be cultured in order to study the role of TH2-like responses in protective immunity. Crude antigen preparations and, later, purified and recombinant antigens will be employed. Cytokine profiles will be examined. The examination of IgE and IgA responses to synthetic peptides and recombinant antigens will include glutathione-S-transferase, paramyosin, calpain and schistosomulum release products.
* Attempts will be made to produce quantities of the 22kDa antigen for use in ELISA assays. λGT11 libraries will be screened with the existing human serum bank to identify other peptides recognised by human IgE. Other native antigens will be identified and purified.
* The relationship of nutritional status with morbidity will be addressed by measuring anthropometric indices, physical performance, school attendance, biochemical indices and ultrasound morbidity in schoolchildren by comparing the effects of immediate versus delayed treatment. After schistosomiasis chemotherapy, a group receiving malaria chemoprophylaxis will be compared with a placebo group: parasitological, clinical and immunological profiles will be followed up before and after the malaria transmission season.

Expected outcome

⇒ Improved knowledge of the relative contributions of Th1 and TH2 cells, and IgE and IgA antibodies to resistance to *S. mansoni*.

⇒ Evaluation of existing, and identification of new candidate vaccine antigens.

⇒ Evaluation of the interaction of *S. mansoni* related morbidity with malaria, nutritional status and other factors.

Discussion and conclusions

The results have been obtained confirm and extend the observations made during previous phases of the STD programme on immune responses in human *schistosomiasis mansoni*. In particular, they are compatible with the hypothesis that protective immunity is associated with Th2-regulated responses, especially IgE production, while such responses are also associated with a relative lack of severe morbidity.

The antigens recognised by those IgE antibodies that are correlated with the expression of immunity have been cloned and characterised, and appear to consist of a group of structurally related molecules, in particular Sm22 and Sm12 (a fragment of another molecule, Sm21.7).

These molecules, which contain potential calcium binding domains, are present in the tegument of the developing schistosomulum and adult worm, as well as the gut and certain other structures such as flame cells.

However, although Sm22 is released into the supernatant of cultured lung-stage schistosomula, it is not expressed at the surface. It has not yet been demonstrated conclusively that these molecules are targets, rather than simply markers, of protective immunity: if they are indeed targets, it is not clear what immune effector mechanisms might be involved. One possibility is that, following release from the organism, they bind onto its surface, thus forming a target for eosinophil-mediated ADCC reactions: another, more simple explanation, is that they elicit immediate hypersensitivity reactions in the lung or liver that lead to reduced parasite viability.

In contrast to protective immunity, the expression of severe morbidity is associated with the production of TNFα, and to a lesser extent IFNγ, in response to egg antigens: Th2 responses with IL-5 production are associated with a lack of morbidity. Thus it would appear that, in contrast to the mouse model of *S. mansoni* infection, Th2 responses in humans are desirable, in that they are associated both with protection and with lack of morbidity. However, the factors that control the development of such responses remain uncertain, and one important area for future study is the question of whether the slow development of protective IgE antibodies in older children and adults depends on the actual duration of exposure to appropriate schistosome antigens, or more simply on age-related physiological changes. Studies to address this problem have already been initiated in an area of recent immigration. Future studies will also concentrate on the role of genetic factors in governing the developing of protective Th2 responses and pathogenic predominant Th2 response in the context of other infections.

In the meantime, schistosomiasis remains a problem of public health importance in Kenya, whose control through chemotherapy has continued. Our recent observations that children without severe clinical manifestations of disease may still show improved physical growth and enhanced examination performance following treatment of their schistosome infections indicates that even mild infections should be considered to be of importance in the context of the development of the school age child.

Contract number TS3*CT920066

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Contract number TS3*CT920117

EPIDEMIOLOGY AND TRANSMISSION DYNAMICS OF SCHISTOSOMA INFECTIONS OF THE *S. HAEMATOBIUM* GROUP IN MALI, SENEGAL AND ZAMBIA

Period: January 1993 - June 1996

Co-ordinator: FACULTEIT DIERENGENEESKUNDE, LABORATORIUM VOOR PARASITOLOGIE EN PARASITAIRE ZIEKTEN, Merelbeke, Belgium (J. VERCRUYSSE)

Objectives

The central objective of the project is to define the ecological and genetic factors snail and parasite which determine the focal levels of endemicity of Schistosoma infections of the *S. haematobium* group in Mali, Senegal and Zambia.

Activities

* In Mali, levels of infection and re-infection were determined from 5 selected villages (high and low endemicity, mixed and pure infections; 200-300 individuals each) which were not covered by the Government control programme. Parasitological and circulating *Schistosoma* antigen detection studies were determined before chemotherapy and at 6 weeks, 1 year and 2 years after treatment with praziquantel.
* Water contact observations were made in the sites where villagers have their main water-contact activities. These observations were carried out by local observers, working from six in the morning until six in the evening, for three days every two months. Malacological studies including bi-monthly snail sampling, identification and snail shedding were also completed.
* In Senegal, the prevalence and intensity of *S. haematobium* and *S. mansoni* infections were determined in 5-14 year old children in a series of villages in the Lower Valley and Middle of the Senegal River Basin (SRB). Snail-parasite inter-relationships were investigated, and physical/chemical characteristics of snail habitats and transmission foci were analyzed.
  In Zambia, schistosomes of the terminal spined egg-complex (*S. haematobium*, *S. mattheei*) and their intermediate hosts were identified and their interactions in cattle and humans were studied. In addition, the potential of the recombinant *S. bovis*-derived glutathione-*S-transferase* (GST) to protect cattle against *S. mattheii* infections was tested.
* Parasite identifications were on the basis of egg morphology, enzyme (isoelectric focusing electrophoresis) and DNA (randomly amplified polymorphic DNA assay) analysis. Isolates of parasites were used for studying snail susceptibility.

Results

In Mali, a total of 1262 individuals have been registered from the 5 villages: Boro, Kassa (Dogo Country), Rigandé, Siguivoucé (Office du Niger), and Massabla (Sikasso Region).

The results showed that only *S. haematobium* and *S. mansoni* are present in Mali.
S. haematobium was the most prevalent species and was hyperendemic in the Office du Niger region (prevalences of 77% and 75% in the villages of Rigandé and Siguivoucé, respectively: with more than 20% of heavy infections: i.e. >50eggs per 10 ml urine), hyper or hypo-endemic in Dogon Country (77% and 27% in Boro and Kassa, respectively), and non-endemic in Sikasso Regino (>1% in Massabla). Children 7 to 14 years old were the most infected age group. Apart from the Office du Niger, where high endemicity was observed with more than 55% of heavy infections, *S. mansoni* infections were rare. No evidence for current infections with *S. intercalatum* was found. Treatment with a single dose of praziquantel significantly reduced for up to two years the prevalence and the intensity of the disease in endemic areas.

No difference was observed between the results of parasitological and serological infection detection methods in villages with high level of endemicity, whereas in those with moderate level of infection the serological prevalence was significantly higher than the parasitological prevalence.

The CAA test on serum provided a more accurate assessment for both the prevalence of schistosomiasis and the impact of chemotherapy. In Office du Niger, highest prevalence was obtained with the urine-CCA assay, and this test was found to be the most sensitive in diagnosing either a single *S. haematobium* or *S. mansoni* infection, or a mixed infection. Transmission studies showed that water contact activities were very varied and intense in Dogon Country and Office du Niger, contrary to Sikasso Region. The most frequent types of water contact observed were: washing of clothes/utensils/vegetables, swimming and filling of wells. *Bulinus forsalii*, *B. truncatus* and, very rarely, *Biomphalaria pfeifferi* were collected from various sites.

In Senegal, 7750 people were examined from the 9014 who were registered from 180 villages and 4 towns. *S. mansoni* was found in the lower valley (Lower Delta - Senegal River, Lower Delta - Lampsar River, Upper Delta and Diéré) but not in the Middle Valley. The mean prevalence ranged from 4.4% in the Lower Delta - Senegal River to 71.8% in the zone of Lac de Guiers, where prevalence and intensity of infections were higher on the eastern side of the lake (81.3% with a mean of eggs/g of 2088egg/g faeces) compared with the Western side (50.3% with a mean of eggs/g of 1,111). *S. haematobium* was recorded throughout the area of study, ranging from a mean prevalence of 0.37% in the diéré of the Lower Valley to 41.5% in the Lower Delta - Lampsar River where the mean egg count was 313 eggs/10 ml urine. Adult schistosome worms were collected from infected sheep and cattle at Dakar and St Louis abattoir in Senegal (n = 313). Examination of these worms by isoelectric focusing showed that all schistosomes from Dakar were *S. curassoni*, and almost all worms from St Louis were *S. bovis*. Rare cases of hybrids were observed in worms recovered from cattle at St Louis abattoir, which is good evidence of the occurrence of natural hybridization between *S. curassoni* and *S. bovis*. In Senegal, *Biomphalaria pfeifferi*, *Bulinus senegalensis*, *B. globosus*, *B. umbilicatus*, *B. truncatus* and *B. forskalii* were collected. Snail infection experiments suggested the existence of three distinct strains of *S. haematobium* in the Senegal River Basin, each with a distinct intermediate host specificity: one *B-globosus* borne and one *B-senegalensis* borne in Senegal, and one *B-truncatus* borne in Mali.

In Zambia, parasitological surveys were conducted in school children in Chisamba (rural) and Lusaka area (urban). The prevalence of *S. haematobium* infection was 56.7% (n = 141) in Chisambe and 21% (n = 235) in Lusaka. No *S. mansoni* infection was observed. The presence of polymorphic shaped eggs in urine samples suggested possible hybridization between *S. haematobium* and *S. mattheei*. Adult schistosome worms were collected from cattle at slaughterhouses in Lusaka (n = 542). Examination of these worms by isoelectric focusing electrophoresis showed that S. mattheei was the most prevalent species (75%), followed by *S. leiperi* (12%) and *S. margrebowiei* (2%). Interestingly, the remaining 11% of worms gave two distinct heterozygote patterns, indicating possible interactions between *S. haematobium* and *S. mattheei* in mixed infections revealing the existence of a mate preference for each of the two species.

S. haematobium exhibited a greater specific mate recognition system than does *S. mattheei*, and male *S. haematobium* were better at pairing with female worms than *S. mattheei* males. Cross infection experiments showed the viability of the hybrids from *S. haematobium* male *X S. mattheei* female, and their ability to develop in sheep. Although the experimental protocol was designed to produce both reciprocal crosses between the species of schistosomes, the reverse cross, i.e. *S. mattheei* male *X S haematobium* female was not obtained. This cross is apparently not viable. Interestingly, snail studies suggested that *B. africanus* is not present in Zambia, and that only *B. globosus* is commonly involved in transmission of *S. haematobium* and *S. mattheei* in this country.

A vaccination study showed that in natural infections protection against *S. mattheei* infection in cattle can be induced by vaccination with the rSb28GST. The induced immunity affects both the worm viability and the accumulation of eggs in the host tissues, and significantly reduces the excretion of viable eggs.

Conclusions

The completion of epidemiological surveys of schistosomiasis showed that the prevalence and incidence of both urinary and intestinal schistosomiasis are increasing in parts of the Senegal River Basin at an alarmingly rapid rate. The distribution and the levels of endemicity are highly focal, due to the diversity in ecology, the local water development projects, the behaviour and the activities of the population. The principal cause for this increase of schistosomiasis is the construction of the dams at Diama and Manantali on the Senegal River, together with the creation of new irrigation schemes. Because of the dams, sea water no longer enters the river beyond Diama, and consequently intermediate host snails are able to multiply dramatically. It is of importance that this changing situation is carefully monitored over the forthcoming years, as such information is of relevance to the implementation of control programmes.

The circulating Schistosoma antigen detection assays were found to be useful for determining rates of infection and efficacy of chemotherapy. Based on parasitological examination, it can be very difficult to distinguish re-infection from failure of cure, especially in areas where transmission levels are high, as viable eggs may still be found in the excreta for several weeks following chemotherapy. Since it takes several weeks before CAA and CCA can be detected after the establishment of a new infection, a rapid decrease of antigen levels following successful treatment would therefore make circulating antigen determination an interesting alternative. Our results showed that serum CAA detection was at least as effective as urine examination for determining prevalences of *S. haematobium* infection in a highly endemic village, and it was better than parasitological examination in a moderately endemic village.

In a mixed *S. mansoni*/*S. haematobium* area it was shown that before treatment and each survey after treatment, the highest percentages of positives were found with CCA detection in urine. Sensitivity of a single urine CCA assay was found comparable with one stool and two urine examinations, but preliminary results suggested that sensitivity in mixed infection areas of low endemicity was low. In a non-endemic area the assays were viewed as useful complementary tools in the detection of infections. Although further improvement of the lower detection limit and simplification of the assay will be needed to make it applicable for mass screening, it is useful in its present form for research purposes and monitoring sentinel populations. However, due to the costs involved with the production of monoclonal antibodies, continuous material and financial support will be needed for their routine use in the control programme. Interestingly, the study showed that the assay can be performed adequately in a regularly equipped public health laboratory within a developing country.

A reassuring finding of the effectiveness of chemotherapy was the reduction of prevalence and intensity of infections, for at least two years. Since the development of pathology (e.g. fibrosis) is related to high intensity of infection, it follows that reduction of infection, which was remarkable in one village of Dogon Country, Boro and the two villages of Office du Niger in this study, will prevent progression of pathology. For control of the disease, the policy of the NSCP was applied, i.e. mass treatment when the prevalence of infections is >40% and/or the prevalence of heavy infections >5%. The strategy proved to be efficient in controlling the prevalence and intensities of infections, but needs also to be evaluated on the longer term, not only with regard to epidemiological impact, but even more with reference to feasibility, affordability and sustainability within the existing health system.

Training activities were an important component of this STD3 project.

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Contract number TS3*CT930237

MARKERS OF MORBIDITY IN *SCHISTOSOMA HAEMATOBIUM* INFECTION

Period: January 1, 1994 - December 31, 1996

Co-ordinator: DANISH BILHARIASIS LABORATORY
Charlottenlund, Denmark (B.J. VENNERVALD)

Objectives

- To evaluate and further develop the ECP (Eosinophil Cationic Protein) assay for use in morbidity assessment in *Schistosoma haematobium* infection.

- To develop and evaluate assays for *S. haematobium* egg antigens in urine with particular reference to protein antigens released by eggs present in the urine and bladder tissue opposed to circulating polysaccharide antigens excreted through the kidney.

- To assess the effect of treatment and reinfection on urinary tract pathology among school children in two endemic areas using the non-invasive assays detecting ECP and *S. haematobium* egg antigen in urine compared to ultrasonography findings and conventional diagnostic methods.

- To train Kenyan and Tanzanian investigators:

  — in laboratory techniques for ECP and schistosome antigen detection;

  — on ultrasonography including assessment of intra- and inter-observer variations as means of detecting urinary tract pathology in *S. haematobium* infection.

- To transfer the developed non-invasive assays to Kenya and Tanzania.

Activities

* Further development and adjustment of the ECP assay and validation of the assay for use in morbidity assessment of *S. haematobium* infection.

* Development and evaluation of assays for *S. haematobium* egg antigens in urine.

* Longitudinal field studies in Kenya and Tanzania including pretreatment and short and long term follow-up examinations after treatment with assessment of the effect of treatment and reinfection on urinary tract pathology among school children.

* Training and technology transfer.

Contract number TS3*CT930237

Expected outcome

Availability of the ECP ELISA assay and possibly an ECP reagent strip suitable for use in *S. haematobium* endemic areas and assessment of the perspectives of using ECP in urine as a morbidity marker.

Increased information about the extent to which the degree of eosinophiluria in *S. haematobium*-infected children reflects inflammatory activity in the bladder and predicts development of pathological lesions in the urinary tract.

Availability of a *S. haematobium* egg antigen detection assay as a seroepidemiological research tool for research groups in endemic areas.

The ECP and egg antigen assays will provide new non-invasive tools for evaluation of chemotherapy effect and morbidity in urinary schistosomiasis.

The successful transfer of the ECP and antigen detection assays to Kenya and Tanzania.

New knowledge concerning the time interval after which pathology reappears in *S. haematobium* reinfection after treatment.

Additional information on uropathy changes due to *S. haematobium* in on-going control programmes.

Results

All urine samples (approx. 3000 samples) from the Kenyan study have been analyzed for ECP and the samples from Tanzania are currently being processed. Data analysis is in progress and results obtained so far show a good correlation between levels of ECP and egg counts both pre- and post-treatment. The diurnal variation in ECP levels is much less pronounced than the variation in egg counts or hematuria. These is a considerable day-to-day variation in the urinary levels of ECP but an ECP level of more than 5 ng/ml in urine has a higher diagnostic value with respect to diagnosing *S. haematobium* infection compared with a single egg count. ECP in urine has been shown to be highly stable with less than 10% reduction in ECP content after storage at 25°C for 24 hours and no reduction after storage at 4°C or below.

A sensitive and specific enzyme linked immunosorbent assay for detection of soluble *S. haematobium* egg antigens in urine have been developed.

Positive correlations between antigen levels in urine (ng/ml) and egg counts (eggs/10 ml urine) have been demonstrated.

Urine samples from the Kenyan field study are currently being tested and diurnal and day-to-day variation and correlations with morbidity will be assessed.

The field work in Kenyan and Tanzania has now been completed with an 18 month post treatment follow up study in Kaloleni district, Kenya and a 2 year follow up in Mikumi,

Tanzania. Out of the initial study population of approximatively 500 children in each country, 296 children from Kenya and 232 children from Tanzania were present throughout the study for 6 or 7 examinations respectively.

The data from Tanzania show a sharp decline in urinary tract pathology within the first 6 month post-treatment from 76% of children having pathological lesions before treatment to only 11% pathological lesions at six month post treatment despite reinfection. Furthermore these data show that urinary tract pathology reappears in some but not all children who showed pathology before treatment.

Two medical doctors, one from Kenya and one from Tanzania, have been trained in ultrasonography. Two Kenyan technicians and 2 Tanzanian technicians have visited Tanzania and Kenya respectively during the field surveys. A Kenyan scientist is based at the Department of Parasitology, University of Leiden, working on a PhD project. A workshop on "Morbidity assessment in schistosomiasis" was held in Mombasa November 1995 with the participation of all project collaborators for presentation of preliminary results.

The ECP assay is currently being adapted for field use. It will be transferred to Tanzania and Kenya in due course.

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Contract number TS3*CT930248

THE EPIDEMIOLOGY, IMMUNOLOGY AND MORBIDITY OF *SCHISTOSOMA HAEMATOBIUM* INFECTIONS IN DIVERSE COMMUNITIES IN ZIMBABWE

Period: January 1, 1994 - December 31, 1997

Co-ordinator: UNIVERSITY OF GLASGOW,
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Glasgow, United Kingdom (G. COOMBS)

Objectives

- To examine patterns of infection and reinfection with *S. haematobium* in communities in Zimbabwe in relation to their (diverse) transmission dynamics.
- To relate levels of infection and reinfection with *S. haematobium* to serological and cellular reactivities to parasite antigens and to observed patterns of exposure to infection.
- To allow the testing of hypotheses regarding the role of cellular responses and antibody responses in the development of human resistance to *S. haematobium* infection.
- To describe *S. haematobium* related morbidity and its resolution following chemotherapy.
- To examine the effects of repeated chemotherapy on the development of pathology and resistance to infection.
- To extend and improve immunological and mathematical modelling expertise at The Blair Research Laboratories, Zimbabwe.

Activities

The initial phase of the study involved parasitological surveys or several locations in Zimbabwe to identify areas of low and high prevalences of *S. haematobium* infection. The aim was to re-examine the issue of the inter-relationship between infection levels immune responses and age. This is likely to be a complicated relationship since infection levels and immune responses are interdependent and both may be related to exposure to infection and possibly also to age. Observations of a "peak shift" in the age prevalence and intensity curves, with children in high transmission areas acquiring higher prevalences and intensites of infection at a younger age than those in areas of lower transmission raised the possibility that differences might also exist in the evolution of the immune response in areas where infection patterns differed. Investigation of such a situation could help distinguish between past history of exposure to infection and the effects of age.

Once the areas of high and low prevalence were identified urine samples were collected for *S. haematobium* egg counts prior to treatment with praziquantel. Serum samples were collected to allow measurements of antibody levels and peripheral blood mononuclear cells were collected for measurement of *in vitro* cytokine production in response to stimulation with antigens and mitogens. Parasitological and serological measurements were repeated at intervals after chemotherapy. Ultrasound was used to assess levels of pathology. Some of the results of this study are described below.

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In a separate area a cohort of children were identified for an investigation of repeated chemotherapy with Praziquantel. In this cohort parasitological and serological assessments are repeated at two-monthly intervals. Exposure to infection continues but adult worms are eliminated or are held at low levels by the repeated chemotherapy.

The intention is to examine the evolution of serological reactivity in a group encountering repeated exposure to larval *S. haematobium* in the relative absence of exposure to adult worms and egg antigens. This study is ongoing. Parasitological and serological samples have been collected but the serum samples have yet to be analysed for antibody responses to worm, egg and larval antigens.

Pre-intervention Parasitology and Serology

Antibody responses to *S. haematobium* worm and egg antigens were measured by ELISA in 133 people from the low infection area of Kaswa and of 147 people from Valhalla. In keeping with the pre-intervention observations prevalences were 33.8% and 62.7%. Overall infection intensities were significantly higher in the high infection area and both prevalences and intensities demonstrated a "peak shift" with the prevalence of infection peaking at 8 years of age and at a higher level than in the low infection area where peak infection levels were not attained until 21-25 years of age. Those from the high infection area produced significantly more IgE and IgG3 against SEA and WWH. They also produced significantly lower.levels of IgA against SEA and IgM against SEA and WWH. Females produced significantly more IgG1, IgG4 and IgM against soluble egg antigens (SEA) and IgE and IgG1 against whole worm homogenate (WWH). From these data it is impossible to comment on whether or not the antibody responses which have been measured have a protective function. Nevertheless the results illustrate marked differences between the two areas in the beahviour of the age-prevalence and age-intensity curves with evidence of a peak shift which would be consistent with the acquistion of an acquired immunity to infection. Furthermore, antibody responses differ between the areas and are slower to develop and lower in the area of low infection.

Reinfection

Antib ody responses have yet to be analysed in relation to reinfection levels. Reinfection post-chemotherapy with praziquantel followed the expected patterns, given the pre-selection criteria of high and low prevalence of infection. After chemotherapy infection levels in both areas fell below 4% but by 8 months after treatment infection prevalences had reached 50% of pre-treatment levels.

Pathology

Bladder abnormalities were detected in only 0.6% of people pretreatment. After treatment no bladder wall abnormalities were detected. Similarly right kidney abnormalities were detected in 5% of individuals pretreatment reducing to 1.9% after treatment.

Contract number TS3*CT930248

Cytokine responses to infection

Using the supernatants obtained from the short field trip to an S .ahaematobium endemic area The Gambia. Assays for IL-2, IL-4, IL-5, IFN-γ, TNFα and GM-CSF were established. Analysis of the data revealed that infected people irrespective of age had higher peripheral blood eosinophil counts ($p<0.0029$) and produced more IFN-γ on stimulation with soluble egg antigen (SEA) ($p<0.0166$) and whole worm homogenate ($p<0.02$) than uninfected people.

Uninfected people had a greater capacity to produce IL-4 ($p<0.0007$) and produced more IL-4 on stimulation with SEA ($p<0.0472$). No significant differences in GM-CSF, IL-2, IL-5 TNFα or cell proliferation were found between children and adults, or infected and uninfected groups. These results were promising. It appeared that the assays which have been established in the laboratory are sufficiently accurate and sensitive to detect the low levels of cytokine production in the supernatants from antigen stimulated peripheral blood cell cultures. Additional work has been done on the comparison of the ELISAs with bioassays and we are now confident that the ELISAs are detecting predominantly biologically active cytokine. These results are important for other reasons, they support the hypothesis that Th2-type responses may have a protective role against schistosomiasis in humans, one of the basic hypothesis being tested in the current survey.

The cells collected from the surveys in Zimbabwe from August to October 1994 were defrosted and used in stimulations and cytokine assays. Preliminary studies were done on peripheral blood cell samples from individuals who failed to provide all the necessary parasitological samples for inclusion in the study or provided blood samples which would be insufficient to provide enough cells for the necessary stimulations. Cytokine production could not be detected in supernatants from these antigen stimulation of these cells. A whole blood stimulation assay was then tested in the field and proved to be more successful. The patterns of cytokine production from these cells are now being analysed.

Water contact

The most striking observation made during the study of water contact activities is that the water contact of children, under five years of age was observed frequently. Follow-up of this observation revealed high levels of contact occur even in children under two years of age. This contact is likely to be significant in terms of the immunology and epidemiology of *S. haematobium* infection.

Contract number TS3*CT930248

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Contract numberTS3*CT940330

MODELLING FOR THE CONTROL OF SCHISTOSOMIASIS AND OTHER EXTENSIONS OF CEC/STD SCHISTOSOMIASIS RESEARCH NETWORK ACTIVITIES

Period: November 1, 1994 - April 30, 1996

Co-ordinator: RIJKSUNIVERSITEIT LEIDEN, DEPT. OF PARASITOLOGY
Leiden, The Netherlands (B. GRYSEELS)

Objectives

- The project aimed at complementing ongoing research activities on schistosomiasis within the EC/STD3 Network, with particular reference to the following subjects:
- To develop, assess and apply epidemiological simulation models for the planning, monitoring and evaluation of schistosomiasis control interventions, with particular reference to integration in basic health structures, and to immuno-epidemiology and vaccinology.
- To study and compare by ultrasound the dynamics of intestinal and hepatosplenic morbidity due to *Schistosoma mansoni* infection in intense Sub-Saharan foci.
- To study by novel DNA-based technology the relation between HLA (-D) type and susceptibility to infection and disease.
- To investigate factors contributing to reduced drug efficacy in schistosomiasis patients.
- To further extend and strengthen collaboration between epidemiological research groups in developing countries and Europe, and their interaction with operational health programmes.

Activities

Modelling

* With microsimulation and other techniques, submodels were developed to describe and quantify the different steps in the epidemiology and control of schistosomiasis. The submodels were integrated in a comprehensive, practically applicable simulation model.
* The first efforts were concentrated on the interpretation of egg counts as a reflection of parasite burdens. This has led to important new insights, and the development of several practical tools (pocket charts) which allow the interpretation of survey results much more accurately than was possible previously. The result is critical for the eventual success of the simulation efforts.
* A preliminary computer simulation model (SCHISTOSIM) has been developed for the epidemiology and control of *S. mansoni* infection, with the aim of evaluating and predicting the effects of different control strategies. By simulating a series of surveys and treatment, short-term effects of the program were satisfactorily explained by the model. However, long-term predictions do not yet fully match the observed data.
* A major outcome of the simulation exercise is the identification of many gaps in our knowledge of the epidemiology and control of schistosomiasis. As such, the modelling exercise is a rich source for clarifying old and new research questions. A link to qualitative public health models (vertical analysis) has been established.

Morbidity evaluation by ultrasound

* With portable ultrasound technology, morphometric evaluation of intestinal wall enlargement and mesenteric echogenicities in patients with *Schistosoma mansoni* infections were performed in intense foci in Senegal and Uganda.

* In these foci, hepatic lesions by ultrasound in patients with (heavy) *S. mansoni* infections were defined and graded; proposed standard systems were evaluated and optimized.
* Sonomorphological findings were compared with clinical, anamnestic, parasitological and serological parameters and evaluation of simple PHC-approaches to the diagnosis of severe pathology. Parallel studies on this issue were also performed in Tanzania.
* Morbidity findings were compared between a recently infected focus (Senegal) and a chronically endemic focus (Uganda), in order to explore the evolution of chronic morbidity.
* Preliminary investigations of the impact of praziquantel treatment on morbidity in these foci were performed.

The results of ultrasonographical studies confirmed the surprisingly low hepatosplenic pathology (despite the high intensities of infection) in the recently established focus of *S. mansoni* in Northern Senegal, already shown by clinical examination in previous studies.

Preliminary data showed that early antischistosomal treatment not only lowers egg output, but also reduces early and mild hepatic morbidity despite the fact that it does not prevent reinfection.

The study allowed the optimisation of the ultrasound method and revealed a potential new application of ultrasound for assessing the intestinal schistosomiasis pathology. It also raised the need to revise the current WHO grading systems. Comparison of overall morbidity patterns in five different countries suggested a geographical variation of *S. mansoni* induced hepatic lesions in Africa. In East Africa, severe pathological status was recorded more frequently than in West Africa, for comparable endemicity levels.

HLA and susceptibility to infection and disease

The aim of this component was to use novel DNA-based technology to compare the structures of antigen-presenting molecules of the HLA-D subgroup in schistosomiasis patients grouped according to differences in protection from intense infection and in the manifestation of disease, with as aims:

* To identify individuals or groups of individuals at risk for intense infection and severe pathology.
* To provide the basis for characterizing the T-lymphocytes involved and for approaching the analysis of parasite antigens which naturally elicit the critical T-lymphocyte responses. This project component aimed mainly at exploring possibilities and setting up methodological and collaborative mechanisms.

The findings from the HLA studies argue in favour of a role of one or several genes located in the MHC region in the control of infection with *S. mansoni*. Additional studies using refined phenotypes are still in progress.

The studies on cellular immune responses showed that active infection with schistosomes is associated with down-modulation of T cell proliferative responses, and that major differences exist between children and adults in IgG4 and IgE responses. These results clearly provide an exciting perspective for extension to the particular *S. mansoni* areas in Senegal and Uganda, and their relation with morbidity and HLA-susceptibility studies. This will, however, require the establishment of adequate logistic conditions for cellular work in these areas.

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Praziquantel susceptibility

The studies of praziquantel efficacy repeatedly and convincingly confirmed early field observations of low cure rates of praziquantel treatment in the *S. mansoni* focus in Northern Senegal, even with the use of a higher dosage of drug (2 x 30 mg/kg).

Interestingly, the parasite strain was fully susceptible to oxamniquine, which could therefore be seen as an alternative drug. This problem may have implications for world-wide control of schistosomiasis. Further collaborative studies are continuing to elucidate the mechanism of drug-resistance.

Outcome

These types of "gap-filling" projects have proven to be highly productive in the framework of collaborative network research. The various components of this project have all generated exciting and relevant new knowledge.

⇒ Basic egg count models, practical applications and preliminary comprehensive simulation model.
⇒ Preliminary data on HLA vs. infection and establishment of more extensive study protocol.
⇒ Development of ultrasound protocols for intestinal morbidity, and standardised, comparative assessment of intestinal and hepato-splenic morbidity in different areas and types of foci.
⇒ Confirmation of reduced praziquantel efficacy in the Senegal *S. mansoni* focus.
⇒ Establishment of a consensus building for a methodological and multidisciplinary approach to elucidate the mechanisms of praziquantel-resistance in *S. mansoni.*
⇒ Increased network interactions, exchanges, training and capacity transfer.

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Contract number IC18*CT960041

THE DEVELOPMENT OF A CONTROL STRATEGY FOR SCHISTOSOMA HAEMATOBIUM IN THE SENEGAL RIVER BASIN

Period: October 1, 1996 - September 30, 1999

Co-ordinator: UNIVERSITY OF GENT, DEPARTMENT OF PARASITOLOGY, Merelbeke, Belgium (J. VERCRUYSSE)

Objectives

- To carry out an up to date assessment of the current situation on prevalence and morbidity of urinary (and intestinal) schistosomiasis in the Lower Middle and Upper parts of the Senegal River Basin (SRB) and identify new emerging endemic foci.
- To determine the factors which influence seasonality of transmission in Senegal and Mali. This information will be highly relevant in assessing the best time for intervention procedures.
- To determine the correlation between the development of immunity to *Schistosoma haematobium* infection and exposure to infection, in areas with different types of transmission (short / long) and in different types of foci (old / recent).
- To determine the effects of agriculture and water development on the spread of schistosomiasis in the SRB.
- To evaluate reinfection patterns and immunity development according to different timings of treatment.

Activities

* A survey will be carried out in the Upper part of the SRB (Bakel, Manantali) to assess the current situation on prevalence and morbidity of schistosomiasis and the compatibility of *Schistosoma haematobium* with the local intermediate hosts (transition zone?). Suitable villages in Mali will be selected for the further studies.
* Epidemiological, morbidity and control studies. During a two year period longitudinal immuno-epidemiological studies will be carried out in 2-3 villages in the department of Podor (seasonal transmission), in 2-3 villages in the department of Dagana (long transmission period) and in 2-3 villages in the Upper Valley around Manantali (periodicity of transmission still to be determined). Before and after treatment, the current situation will be assessed by blood, faecal and urine sampling procedures, clinical examination and echography. Follow-up will be done in relation to timing of initial treatment (before the rainy season).

Immunological studies. Serum isotype analysis of specific human antibodies will be carried out (with emphasis on the IgA response to rSh28GST) and the functional properties of the antibodies (capacity to inhibit GST enzymatic activity) will be assessed. A comparative analysis of the immune responses between populations living in old or new foci, the evolution of the immunological parameters according to the seasonal or perennial transmission type, and the influence of treatment upon the acquisition of immunity to subsequent exposure will be investigated in each selected cohort.

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* Transmission studies. Longitudinal malacological studies will be carried out during two years in the selected villages where the transmission sites will be identified. Sampling will be conducted at monthly intervals in Senegal and three-monthly intervals in Mali. Further laboratory studies will characterise fully the compatibility of the collected snails with the parasite species (strains) naturally transmitting in the SRB. These procedures will involve SEM, IEF and RAPD techniques. Physico-chemical characteristics will be measured over time from a number of transmission foci in order to assess the predictability of the likely outcome of potentially newly created transmission foci associated with agricultural development.

Expected outcome

It is expected that the data generated from these studies will give a clear lead as to the most effective control strategy for schistosomiasis in the Senegal River Basin.

These studies will generate important information for future vaccination trials, as it is possible to adapt vaccine formulation (presentation, type of vector) to the different transmission patterns in the field.

It is anticipated that the south-south axis will be strengthened through regional cooperation.

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Contract number IC18*CT960112

IMMUNO-EPIDEMIOLOGY, CHEMOTHERAPY AND CONTROL OF SCHISTOSOMA MANSONI IN RECENTLY ESTABLISHED FOCI IN NORTHERN SENEGAL

Period: September 1, 1996 - August 31, 1999

Co-ordinator: INSTITUTE OF TROPICAL MEDICINE,
Antwerp, Belgium (B. GRYSEELS)

Objectives

- To continue and complete ongoing immuno-epidemiological research on the role and development of age-related acquired resistance to (re-)infection with *schistosoma mansoni* in recently established foci in Northern Senegal.
- To investigate the consistently observed poor therapeutic responses to praziquantel in this focus, with particular respect to the possibility of reduced parasite susceptibility and to the interference of underdeveloped immune responses.
- To further investigate the ecological dynamics of this unstable epidemic situation, with particular respect to the spread and vectorial capacity of the intermediate host.
- To develop, evaluate and implement sustainable intervention strategies, focussing on passive case detection and safe water supply; health information systems within the existing health services.
- To follow-up and further document the clinical pathology, community morbidity and public health impact of *S.mansoni* infection in this epidemic focus in its transition to endemicity.
- To further strengthen the research and intervention capacity of the Senegalese team by providing training up to Msc/PhD level in each of the above fields, and to consolidate the established collaborative and network links.

Activities

* In-depth analysis and exploitation of data and samples collected in the previous STD3-project (immunology, epidemiology, diagnostics, chemotherapy).
* Further, well oriented studies in immuno-epidemiology, chemotherapy, morbidity, in newly established foci in the area.
* Documentation in the laboratory and in the field the vectorial capacity of the intermediate host, and the possible ecological and genetic determining factors.
* Breeding a number of isolates of the local *S.mansoni* strain and to test *in vivo*, possibly *in vitro*, their susceptibility to praziquantel and oxamniquine, and to study possible biochemical and genetic markers of resistance.
* Clinical and community-based studies in order to optimise clinical definition and classification of specific intestinal and hepatoplenic disease and morbidity.
* Translation of these findings in optimal decision trees for case-management and disease surveillance at primary, secondary and tertiary health services.
* Masters- and PhD-training to Senegalese researchers and health workers active in this project.

Contract number IC18*CT960112

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Contract number IC18*CT970228

PATTERNS OF PRAZIQUANTEL USAGE AND MONITORING OF POSSIBLE RESISTANCE IN AFRICA

Period: December 1, 1997 - November 30, 2000

Co-ordinator: INSTITUTE OF CELL BIOLOGY, Rome, Italy (D. CIOLI)

Objectives

To establish a network of scientists, field workers and health administrators dealing with the treatment of schistosomiasis, a parasitic disease affecting about 200 million people in tropical and sub-tropical countries. Since treatment is almost universally performed using a single drug, praziquantel, the objective of this Concerted Action is to examine in a global perspective various issues related to praziquantel usage, among which:

- to determine the most cost-effective ways of procuring, distributing and evaluating the drug;
- to compare various local experiences regarding drug dosage and administration schedules;
- to collect information on major side-effects and possibly identify any associated risk factors;
- to compare and evaluate all available data on the suspected appearance of drug resistance in the human population;
- to organise the collection of possible resistant isolates of the parasite, their propagation in the laboratory and their testing in experimental animal models;
- to distribute biological samples to research laboratories in order to study the still largely unknown mechanisms of praziquantel action;
- to identify a number of "reference centres" for the monitoring of drug resistance;
- to formulate policies to assist national health authorities to deal with any occurrence of drug resistance.

Contract number IC18*CT970228

Activities

The initial nucleus of six European and nine African members will hold three general meetings, which will be extended to outside experts and additional participants, as deemed appropriate. Guidelines and protocols for the monitoring of resistance will be developed and field-tested, suspected resistant isolates will be collected and distributed to research laboratories. One or more reference centres will be established in Africa and basic equipment and training will be supplied. Information on drug procurement, costs, suggested delivery schedules and observed side-effects will be distributed to all interested bodies and individuals.

Expected outcome

This study will collect a broad range of data on praziquantel usage to help field workers and health administrators in the planning and implementation of antischistosomal chemotherapy. The controversial issue of drug resistance will be tackled by organising the collection and the analysis of potentially resistant parasite isolates. The use of such isolates for the study of basic mechanisms of drug activity will be pursued. Guidelines will be developed for the most rational use of praziquantel and for alternative strategies in case of resistance.

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Contract number IC18*CT970237

MULTIDISCIPLINARY STUDIES OF HUMAN SCHISTOSOMIASIS MANSONI IN KENYA AND UGANDA: NEW PERSPECTIVES ON IMMUNITY AND MORBIDITY

Period: November 1, 1997 — April 30, 2001

Co-ordinator: DANISH BILHARZIASIS LABORATORY
Charlottenlund, Denmark (B. VENNERVALD)

Objectives

- To study the effect of age as such and duration of exposure on resistance to reinfection with *Schistosoma mansoni.*
- To study the specific IgE responses related to reinfection with emphasis on their regulation at the cellular level and the influence of host genetics on the IgE response.
- To study the immunological responses associated with severe *schistosomiasis mansoni* morbidity with emphasis on their regulation on the cellular level and the influence of host genetics on TNFa production.
- To assess morbidity using ultrasonography and alternative markers and to study how exposure to other infections or hepatic insults affect the severity of schistosome-associated morbidity.
- To assess the effect of transmission blockage on morbidity regression.

Activities

In Kenya an established structure for multidisciplinary field and laboratory studies will be maintained and sets of cohorts are already available for detailed study and longitudinal follow up. Further activities will include:

* Comparison of immune responses in individuals of same age but with different history of exposure including serological studies and assessment of lymphocyte proliferation and cytokine production in response to worm and egg antigens.
* Studies on immune responses associated with immunity and morbidity and any potential linkage with polymorphic microsatellite markers within the Th2 IL-4/5/9/13 gene cluster on chromosome 5.
* Case-control studies with detailed examination of cytokines associated with regulation of Th1-type responses, the ability of PBMC's to release TNF in response to antigen stimulation and other factors involved with the expression of TNF.
* Focal molluscicding has been introduced in Kambu River and the regression of morbidity will be determined in this area where transmission has been blocked.

Studies in Uganda will include:

* Baseline studies with systematic collection of snails, administration of questionnaires concerning nature and time of different water contact activities by age and sex supplemented with direct observations and parasitological examinations.
* A treatment/reinfection study involving 500 individuals randomly selected after age stratification. Reinfection will be followed by stool samples at yearly intervals and blood samples will be collected for serological studies.
* A case-control study involving individuals with or without severe morbidity (as judged by ultrasonography) matched for age, sex and intensity of infection. Comparisons will be made with respect to history of exposure, alcohol consumption, nutritional status,

presence of hepatitis B surface Ag and anti-hepatitis C Abs. Blood and faecal samples will be taken for measurement of inflammatory and collagen markers. DNA samples will be tested for TNFa polymorphisms.

Bayesian statistical theory and Markov Chain Monte Carlo techniques will be applied using the Gibbs sampler to analyse the large databases accumulated from the Kenyan studies.

Expected outcome

The project will widen and deepen the range of scientific skills and techniques of Kenyan scientists and facilitate the transfer of these same skills to scientists in Uganda through a strong South-South collaboration.

In addition, results from this project will lead to improved and refined strategies of the development of anti-schistosome vaccines and the control of schistosome-associated morbidity.

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Presentation of EC supported joint research projects (1991-1996) continued
STD3
INCO-DC: 1st and 2nd Call

Areas of interest:

3. Trypanosomiasis

Contract number TS3*CT910029

A SURVEY OF CHAGAS CYCLES IN URUGUAY BY USE OF GENETIC MARKERS WITH SPECIAL EMPHASIS ON REINFESTATION HAZARDS OF DOMESTIC STRUCTURES BY SYLVATIC CYCLES

Period: April 1, 1992 - March 31, 1995

Co-ordinator: ORSTOM MONTPELLIER I, LABORATOIRE DE GENETIQUE MOLECULAIRE DES PARASITES ET DES VECTEURS, Montpellier, France (J.P. DUJARDIN)

Objectives

To understand why northern and southern areas of Uruguay present different epidemiologically features: domiciliated, more infected insects (*T. infestans*) in the northern departments versus peri-domiciliated, less infected insects in the southern departments.

Triatoma infestans (Hemiptera, Reduviidae) is almost exclusively domestic in northern departments of Uruguay, such as Artigas, Cerro Largo, Rivera and Tacuarembo, but mainly occupies peridomestic habitats such as chicken coops in the southern departments of Soriano or Colonia. These areas differ in median temperature and humidity, with the northern regions tending to be warmer and drier, but also differ in socio-economic conditions such that rural dwellings in the northern departments are usually constructed of wood and/or adobe blocks, whereas brick and cement houses are more common in Soriano. This parallels higher infection rates of *T. infestans* with *T. cruzi* (causative agent of Chagas disease) in the northern departments.

We were interested to examine the environmental and genetic contributions to the observed epidemiologic differences.

To understand the reinfestation mechanisms of treated areas by the original vector species (*Triatoma infestans*), or by another one (*T. rubrovaria*).

Reinfesting populations may represent hidden survivors recovering from the control treatment or they may be bugs immigrating from untreated foci. Operationally, it is important to distinguish between "survivors" (indicating control failure) and "immigrants" (indicating poor geographic coverage). Moreover, localized domestic invasions occurring in Uruguay were due to *T. rubrovaria*, a little-known species of *Triatominae*, providing a certain imperative to identify bug populations that might present a future risk of domiciliation.

This research programme was thus naturally inserted in the activities of the National Control Programme of Chagas Disease in Uruguay.

Main results

(1) First objective

Though they were not distinguishable by isoenzyme electrophoresis, northern and southern populations of *T. infestans* in Uruguay were found to strongly differ at both cytogenetical and morphometrical characteristics. These results were related to the two-ways entry of *T. infestans* in Uruguay. *T. infestans* is believed to have originated from silvatic populations in central Bolivia and to have been dispersed mainly in association with human migrations, particularly during the last century.

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Historical reconstruction suggests that it entered northern Uruguay from southern Brazil at around the turn of this century, but it appears to have entered southern Uruguay from Argentina some 50 years earlier since "vinchucas" (the local term for domestic Triatominae in Argentina and Uruguay) are mentioned in southern Uruguay in the chronicles of W.H. Hudson first published in 1865. Biogeographically, the northern and southern departments of Uruguay are separated by the Rio Negro, which was bridged only a few decades ago, so that the apparent cytogenetical and metrical differences between northern and southern populations of *T. infestans* seems to accord both with their different origins and with an ecological barrier between them. In both cases, we can infer development from original founder populations, and assume that these would have differed slightly due to genetic drift which may or may not have been modified by adaptation to the different environments.

(2) Second objective

The various data obtained - cytogenetic, morphometric and isoenzymatic - converged on the idea that reinfestant specimens arose from a residual population. Indeed, in case of exchange of individuals between departments, between sectionals or between segments (administrative units in Uruguay), it was unlikely to find such an amount of cytogenetic and morphometric differences between them. Furthermore, when comparing reinfestant specimens with the insects collected before insecticide treatment, it was not possible to detect significant differences. The use of blood meal identification brought further, more defined, information about the mechanisms of reinfestation by *T. infestans*, indicating that the residual population was most probably of peridomestic, rather than domestic, origin.

Bloodmeal identification in *T. rubrovaria* revealed that this species had no host preference, indicating that its trend to domesticity was probably not due to an anthropophily, but rather to habitat convenience. Human blood was found in 10% of *T. rubrovaria* specimens, but in peridomiciliary conditions only, indicating that in limited geographic areas this silvatic species could make the link between the domestic and silvatic cycles of the parasite.

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Contract number TS3*CT920075

STUDIES OF THE VACCINE POTENTIAL OF RECOMBINANT INVARIANT SURFACE MEMBRANE PROTEINS OF THE AFRICAN TRYPANOSOMES

Period: October 1, 1992 - September 30, 1995

Co-ordinator: UNIVERSITE LIBRE DE BRUXELLES, LAB. DE PARASITOLOGIE MOLECULAIRE, Bruxelles, Belgique (E. PAYS)

Objectives

The proposal concentrates upon five specific experimental objectives:

- The cloning and sequencing of the EDAG 4/GRESAG 4 genes from *T. congolese* and *T. vivax*.
- The expression and production of the cloned trypanosomal antigens as well as the production of antibodies against these antigens.
- The analysis of the structure, localisation and function of these antigens where this information will either aid the experimental development of the vaccine or help to understand how the vaccine disrupts the physiological function of its target receptor.
- Experimental trials of the pure recombinant antigens and defined segments of these antigens as immunogens and the assessment of their potential for providing protective immunity in experimental animals during subsequent challenge with live parasites.
- The experimental definition of the optimum immunisation protocol in experimental animals.

Activities

Activities can be considered chronologically as taking place within the first, second or third year of the project.

Year 1

* Cloning of the ESAG 4 and GRESAG 4 genes from *T. congolense* and *T. vivax*.
* Selection of appropriate gene fragments and tentative identification of the extracellular domains including effector binding and potential glycosylation sites, the transmembrane spans and the cytoplasmic regions including active and phosphorylation consensus sites;
* Recombinant antigen production using the Baculovirus system;
* Structural analysis, i.e. the extent of glycosylation of the different antigens will be assessed;
* The functional domains of the EDAG 4 protein will be dissected by stable expression of unmodified or mutated versions of ESAG 4 in procyclic forms;
* Sera from infected animals will be assayed for circulating antibodies and antigens;
* Vaccine trials with the purified antigens will study the effect of systematic variation of the immunogen dose, route of administration, immunisation protocol and type of adjuvant.

Year 2

* Subclones of fragments of ESAG 4 will be produced by either restriction endonuclease digestion or PCR amplification;
* Studies of the regulation of chemosmotic potential and ion fluxes;
* Second wave of immunisation trials.

Year 3

* Completion of work on cloning and expression of *T. congolense/T. vivax* genes;

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* Final functional and topological studies;
* Third wave of immunisation trials.

Expected outcome

The following goals should be achieved:

⇒ Genes coding for different adenylate cyclases will have been cloned, sequenced and compared for species of African trypanosomes and protein products made available for use as antigens.
⇒ An estimate of species conservation/divergence will be available.
⇒ Whether these antigens circulate in the bloodstream of infected animals, and the level of circulating antibodies, will have been determined.

Results

⇒ We have cloned and sequenced an ESAG 4-related gene from *T. congolese*. The protein encoded by this gene has been produced in recombinant *E. coli.*
⇒ We have cloned ESAG 4, CRESAG 4.1 as well as mutated versions of ESAG 4 in baculovirus constructs, and used these recombinant viruses to express the trypanosome genes into cells. The role of the different domains of these proteins is under study.
⇒ The localisation of the trypanosome cyclases and their accessibility to antibodies has been studied in detail. The cyclases were found to be shielded underneath the VSG coat.
⇒ Several immunization trials have been performed with antigens produced either in *E. coli* or on the surface of insect cells. Although a good immune response was obtained, no significant protection against challenge infection could be observed.
⇒ Given the lack of protection obtained with the pESAG 4-related antigens, vaccination of mice was attempted with total extracts from the flagellar pocket of the parasite, instead of pure antigens. Two out of three experiments gave positive results. The sera from the protected mice were used to screen a *Trypanosoma brucei* cDNA library expressed in COS7 cells. The clones selected by this approach are presently under study.

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Contract number TS3*CT920092

BIOSYSTEMATICS AND ADAPTIVE TRENDS IN THE GENUS RHODNIUS

Period: February 1, 1993 - January 31, 1997

Co-ordinator: LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE,
DEPARTMENT OF MEDICAL PARASITOLOGY
London, United Kingdom (C.J. SCHOFIELD)

Objectives

This project addresses a problem of particular relevance to Chagas disease control in Mexico, Central America, Venezuela, Colombia, Ecuador, Bolivia, Peru and Brazil, where *Rhodnius* species are important vectors of Chagas disease but, due to morphological similarities between key species, their relative importance and potential for control remains unclear. The project addresses this problem by:

- Clarifying the taxonomic status of members of the genus *Rhodnius* with reference to type and topotype material;
- Defining the geographic limits and ecological characteristics, including relative vectorial capacity, of the main vector species within the genus;
- Assessing the evolutionary trends within the genus, especially in relationship to progressive adaptation to domestic and peridomestic environments.

Activities

This project has established a network of collaborating research partners based in eight Latin American countries with links to research institutions and control organisations in a further five countries. The network provides technical support and coordination, and assistance with field and laboratory work, although most of the research partners also have additional financial support from national sources. All the partners are involved in field work, either as a research activity or in association with vector control services. Several can also act as reference centres providing technical support and reference services to the network in relation to specific analytical techniques and/or statistical analysis of results. Through the network, field collections of different *Rhodnius* species have been made from numerous localities in Mexico, Honduras, Colombia, Venezuela, Ecuador, Bolivia and Brazil. Wherever possible, material has been collected from original type localities, or from regions with similar ecological characteristics. Other species of interest as actual or potential vectors of Chagas disease have also been collected and processed in a similar way. Material is first identified using external morphological characters, and then processed as follows:

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Head: quantitative morphometry; antennae and mouthparts: quantitative analysis of sensilla patterns; salivary glands: gland contents isolated for analysis of protein profiles; thorax: alary muscles fixed for analysis of isoenzyme patterns; legs: analysis of RAPD patterns; wings: analysis of cuticular hydrocarbons (some also mounted for studies of fluctuating asymmetry); gonads: fixed for chromosome studies and karyotype analysis; Genitalia: mounted for qualitative comparisons of cuticular structures.

Results

⇒ Field studies: natural ecotypes of 9 *Rhodnius* species have now been fully characterised, revealing quite specific associations with certain palm species in several cases. However, *Rhodnius prolixus* in Central America seems to be entirely domestic and/or peridomestic while *Rhodnius domesticus* in southern Brazil seems associated primarily with certain types of bromeliad rather than palm trees.

⇒ Laboratory culture: eight of the 13 recognised species of *Rhodnius* have been established in laboratory culture for reference purposed and comparative studies of basic population parameters (reproductive rates, fecundity, survivorship, etc.). These include all species currently considered of epidemiological significance.

⇒ Biosystematic analysis: all species of *Rhodnius* have now been characterised by external genitalia, revealing one new species (*R. stali*) previously classified as *R. pictipes*. Further studies are underway to analyze morphological variation in the main vector species. A system of quantitative sensilla analysis has been developed, and applied to several populations of 6 species, providing useful taxonomic information on pair-groupings and on sensory relationships in relation to habitat specificity. Isoenzyme analysis and head morphometry has been carried out on various populations of 7 species, including all those of greatest epidemiological significance. The results largely confirm the pair-groupings and allow construction of a preliminary phonogram of relationships between species. Preliminary work has been carried out to demonstrate the feasibility of using RAPD analysis for studies of population variability, showing that the technique can be applied to dry-mounted Museum specimens over 50 years old (this opens the possibility of analyzing specimens from regions where they no longer occur). Hydrocarbon analysis has also been carried out on populations of 4 species, showing consistent specific differences and karyotype analysis has been completed for 8 species of *Rhodnius* and 2 species of the closely related *Psammolestes*.

⇒ Within the prolixus group of species, which include those of greatest epidemiological importance, the results show clear differences between *R. prolixus, R. neglectus* and *R. nasutus*, but indicate little if any difference between *R. prolixus* and *R. robustus*. This is also endorsed by results of interbreeding studies involving 14 populations originating from various sites in Central America and Brazil.

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⇒ Training activities: through the network, a number of training visits have been carried out, whereby research staff either receive staff from other institutions for specific training, or travel to other institutions to advise and help resolve technical problems. The aim of these visits is not necessarily to make every technique available in each laboratory, but to ensure that each researcher is familiar with the aims and limitations of each of the techniques being applied (the visits also help with the difficult problem of distributing biological material between laboratories). In addition, a total of 6 post-graduates have been trained and carried out MSc level projects in association with the network research.

⇒ Support for vector control activities: results to date are being used to assist in the design of control strategies and entomological surveillance directed against those species of greatest importance as vectors of Chagas disease. A network workshop was held in September 1995 in Ecuador, during which organizations of several endemic countries participated. One of the most important outcomes is the indication that *R. prolixus* in Mexico and Central America seems to be restricted to domestic and peridomestic habitats, and so far seems to show low levels of genetic variability compared to populations in Venezuela and Colombia. This implies that *R. prolixus* in Central America could be a feasible target for eradication, which would greatly reduce the risk of further transmission of Chagas disease in that region. Elsewhere, domestic populations may be much lower than anticipated, which would also favour renewed emphasis on vector control to reduce Chagas disease transmission. A preliminary report and recommendations has been presented to the control authorities, and the detailed report (including methodological guidelines) is now being finalised for publication.

Contract number TS3*CT920092

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Contract number IC18*CT960042

POPULATION GENETICS AND CONTROL OF *TRIATOMA BRASILIENSIS* IN NORTH EAST BRAZIL

Period: January 1, 1997 - December 31, 1998

Co-ordinator: LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE, London, United Kingdom (C.J. SCHOFIELD)

Objectives

- To improve knowledge of the biology and population genetics of *Triatoma brasiliensis* in support of entomological surveillance and control activities against Chagas disease in North East Brazil, including development of genetic and/or phenetic markers suitable for identifying the source of new infestations.

- To encourage greater collaboration between research scientists and Chagas disease control authorities throughout the arid Caatinga of North East Brazil.

Activities

* Background field work in selected municipalities of Ceara and Piaui

* Establishm ent of laboratory colonies for comparative genetic analysis and studies of population growth rates and flight physiology

* Monitoring of reinfestation rates following trial control interventions using modern pyrethroid insecticides

* Analysis of reinfestant populations to determine population structure and origin

* Technical workshop with control personnel from 8 north-eastern states (Alagoas, Bahia, Ceara, Paraiba, Pernambuco, Piaui, Sergipe, Rio Grande do Norte) to discuss results and refine operational control and surveillance methods.

* Formal presentation of recommendations to Ministry of Health.

Expected outcome

⇒ improved geographic database of *T. brasiliensis* distribution

⇒ establishment of laboratory colonies from well characterised localities

⇒ basic life-table data under different laboratory conditions

⇒ discriminant analysis based on 11-18 morphometric variables and ratios, and on up to 25 gene loci represented by isoenzymes

⇒ preliminary karyotype analysis and RAPD data

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⇒ analysis of geographic structuring of *T. brasiliensis* populations, and preliminary estimates of gene dispersal between neighbouring populations

⇒ detailed characterisation of field trial localities, and liaison with industries interested to participate in field trials

⇒ laboratory flight studies indicating key factors influencing flight initiation

⇒ possible genetic markers for flight capacity

⇒ field studies using light traps to estimate potential flight range

⇒ morphometric and genetic characterisations suitable for analysis of reinfestations

⇒ evaluation of reinfestations following control trials, and reassessment of morphometric and biochemical profiles.

(Note that during the course of this work we also expect to gather preliminary data on other species of Triatominae found in the same region as *T. brasiliensis*, which might become of greater epidemiological significance in the future.)

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Contract number ERBTS3*CT920155

EXPERIMENTAL STUDY OF THE IMPACT OF POPULATION CLONAL STRUCTURE ON RELEVANT MEDICAL AND BIOLOGICAL PROPERTIES OF *TRYPANOSOMA CRUZI*

Period: September 1, 1992 till August 31, 1995

Co-ordinator: ORSTOM, MONTPELLIER 1, LABORATOIRE DE GENETIQUE DES PARASITES ET DES VECTEURS, Montpellier, France (M. TIBAYRENC)

Objectives

- Extensive population genetic analyses have shown that the populations of *T. cruzi*, the agent of Chagas disease, are subdivided into natural clones, stable in space and time. The major clones refer to certain clones which are much more frequently sampled than others; it is suspected that their medical and epidemiological significance is considerable.
- The goal of the present study is to compare some relevant medical properties such as virulence and resistance to drugs of the major *T. cruzi* clones.

Activities

Studies will involve a limited sample of 16 laboratory-cloned stocks representing 3 major clones, selected according to their genetical relationships, ascertained by multilocus isoenzyme electrophoresis. Each major clone in the present sample is represented by several stocks having extremely variable origins (host, place of isolation). Stocks pertaining to these 3 clones have been studied, in order to consider possible interactions between two (or more) different clones. Such mixed infections seem to be common in natural cycles in both triatomine bugs as will as in patients. The impact of clone interactions in a given host could have an important impact on Chagas' pathogenesis. To follow the behavior of such mixed infections, we used the PCR KDNA probes specific for the major clones. Specific study areas will include:

Differentiation in axenic culture medium, which will be estimated from the percentage of trypomastigote forms at given times.

Differentiation of epimastigote forms to infective metacyclic trypomastigote forms will be monitored every day following morphological changes in a Thomas chamber in order to obtain at least 20% of metacyclic trypomastigotes.

Generation of tissue culture trypomastigotes; study of *in vitro* infectivity; experimental mouse infections.

Histopathological studies of the following organs in mice: heart, brain, liver, spleen, ganglion, skeletal muscle and colon.

* Drug sensitivity studies will be carried out on both *in vitro* and *in vivo* models using drugs currently applied in the treatment of Chagas' disease, 5-nitrofuran and 2-nitroimidazole.
* Statistical analysis of the results using commercial software.

Expected outcome

The goal project was to define the medical implications of the considerable genetic variability of *T. cruzi.*

We proposed a general model of parasitic protozoa population structure that strongly suggests that many parasites (e.g. *T. brucei* and various species of Leishmania), have typical clonal population structures, like *T. cruzi*: hence the approach and experimental design proposed could be the basis for further studies involving other parasites.

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Results

A set of 21 parasites stocks representing 4 major natural clones (clonal genotypes that are widespread and constitute most part of the stocks isolated from chagasic patients) have been selected, on the basis of genetic characterization involving 15 isozyme loci.

Since the beginning of the project, the following goals have been reached:
* Improved genetic characterization of the 21 stocks: a more accurate picture of the actual genetic variability of our sample has been provided by:
* increasing the number of isozyme loci from 15 to 23;
* using random primer amplification of polymorphic DNA or RAPD.

During biological characterization of the 21 stocks the following main parameters have been screened:
* Virulence in Balb/C mice.
* *In vitro* culture kinetics (pure clones and mixtures of clones), epimastigote/ trypomastigote transformation rate, *in vitro* drug sensitivity, transmissibility by the vector *Triatoma infestans* (pure clones and mixtures of clones).

All these parameters have been quantified. A highly significant correlation was found between biological variability and genetic diversity: the stocks that are genetically closely related have a strong statistical tendency to have similar biological comportment, while the opposite is the case for the distantly related clones. The major clones tend to behave like distinct taxa for these biological parameters. The stocks attributed to the clonal group 19/20 generally grow faster, transform more actively into trypomastigotes, are more virulent to mice and less sensitive to drugs.

Complementary analyses from anatomopathology and *in vivo* experiments on three different mouse strains confirm considerable biological diversity among *T. cruzi* stocks pertaining to the different major clones.

Conclusions

Most of the work outlined at the start of the project has been completed together with additional experiments on *T. cruzi* transmissibility by *Triatoma infestans* and the biological behaviour of mixtures of clones.

All results fully confirmed the working hypothesis of the project, that clonal diversity of *T. cruzi* has a major impact on this parasite's biological diversity including medically relevant parameters such as virulence, resistance to drugs and transmissibility by triatomine bugs.

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Contract number ERBTS3*CT920130

RISK OF REINFESTATION FROM WILD FOCI OF *TRIATOMA INFESTANS* IN BOLIVIA, A COUNTRY OF THE SOUTHERN CONE PROGRAMME

Period: September 1, 1993 - August 31, 1995

Co-ordinator: ORSTOM MONTPELLIER, LABORATOIRE DE GENETIQUE MOLECULAIRE DES PARASITES ET DES VECTEURS, Montpellier, France (J.P. DUJARDIN)

Objectives

- To evaluate the epidemiological importance of wild populations of *Triatoma infestans* (Hemiptera, Reduviidae). This species is the principal vector of *Trypanosoma cruzi*, causative agent of Chagas disease, throughout the seven southernmost countries of Latin America (Argentina, Bolivia, Brazil, Chile, Paraguay, Peru, Uruguay). In these countries it has become the primary target of Chagas disease vector control programmes.
- To determine the invasive capacity of silvatic *T. infestans*: do they represent a risk for reinfestation?

Activities

Using field experiments and laboratory studies, we studied the connections between domestic and wild *T. infestans*.

Throughout most of its wide distribution, *T. infestans* seems to be exclusively domestic and peridomestic, occupying cracks and crevices in rural dwellings and domestic animal enclosures. True silvatic colonies are known only from the Cochabamba region of southern Bolivia, where the insects can be found amongst rockpiles in association with wild guinea-pigs. The original silvatic focus, some 15 Km south of Cochabamba (Cercado province), was first described in 1946.

A genetic interpretation of electrophoretic data has so far not revealed differences between the silvatic population and nearby domestic populations so that the degree of isolation between them is unclear. However, in order to apply adequate control and surveillance measures, it is important to understand the relationships between these ecotopes. Control of Chagas disease vectors relies primarily on spraying infested dwellings with pyrethroid insecticides. After the initial intervention however, it is important to continue entomological surveillance so that any new infestations can be selectively retreated.

Laboratory studies scored different population markers with the idea that, if not panmictic, the ecotopes under study should show some genetic differentiation. Isoenzyme electrophoresis and cytogenetic studies comparing C-banding polymorphism failed to detect significant differences between ecotopes, whereas random amplified polymorphic DNA (RAPD) evidenced different band frequencies. In accordance with this latter genetic marker, morphometric analysis revealed also head and wing differences. The post-ocular region was repeatedly larger in silvatic specimens, either nymphs, males or females, in samples collected at various times (1983, 1992, 1995 and 1996).

Contract number ERBTS3*CT920130

Field experiments explored the dispersive behavior of wild *T. infestans* in Jamach'Uma (Cochabamba, Bolivia). This locality is a small village surrounded by silvatic foci of *Triatoma infestans*. The first experiment used "sentinel henhouses". Artificial, adobe made henhouses were constructed each 100 meters from Jamach'Uma to the wild focus. Though hens are very attractive animals for *T. infestans*, none were found colonizing these henhouses during a six months survey. Hens were then replaced by rodents, and monthly vigilance was continued for eight months. Again, no *T. infestans* were found, though another species (*T. sordida)* invaded these artificial structures.

The second field experiment simulated a control programme in Jamach'Uma. In December 1992, infestation by a few nymphs of *T. infestans* was found again which was sprayed in October 1993. They were compared at seven head metrics with 36 fifth instar domestic nymphs coming from Jamach'Uma before treatment, and with two sets of nymphs originating from the surrounding silvatic foci: 8 specimens collected in 1992 and 9 specimens collected in 1995. The results were interpreted in terms of the possible mechanisms of reinfestation whether there was a residual population or reinvasion from surrounding silvatic foci. Metric comparisons strongly supported the hypothesis of infestation resulting from a residual population surviving the insecticide spraying. It is not possible to definitively rule out the idea that some of the infestant nymphs are of silvatic origin, mixed with a residual population. Three arguments were consistent with the hypothesis of no regular migrants, or exceptional migrants, between Jamach'Uma and the wild focus: the delay (ten months) between insecticide spraying and the reinfestation, the stage (fifth nymphs) of the infestant specimens and their metric characteristics.

On the basis of these laboratory and field data, the silvatic focus of *T. infestans* in Bolivia does not appear to represent a serious obstacle to the application of the Southern Cone Programme in Bolivia.

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Contract number: ERBTS3*CT930226

ROLE OF INSECT HOST DEFENCES IN TRYPANOSOME DEVELOPMENT IN CHAGAS' DISEASE VECTORS WITH EMPHASIS ON THE ACTIVITY OF IMMUNE DEPRESSION AGENTS

Period: January 1, 1994 - December 31, 1996

Co-ordinator: UNIVERSITY COLLEGE OF SWANSEA, SCHOOL OF BIOLOGICAL SCIENCES, Swansea, United Kingdom (N. RATCLIFFE)

Objectives

- To determine the presence and role of insect vector immune defence factors in insect-parasite interactions.
- To determine the effect of parasites on the vector immune defence reactions.

Activities

Year 1

* Lectin staining.
* Hemolymph and crop lectins purification initiated.
* Infectivity studies on different parasite strains.

Year 2

* Lectin staining.
* Hemolymph and crop lectins purification.
* Effect of immunosuppressive agent.
* Prophenoloxidase.
* Blocking experiments commenced.

Year 3

* Lectin staining.
* Blocking experiments.
* Test pure lectin against parasites.

Exchange of scientists and training.

Contract number: ERBTS3*CT930226

Expected outcome

⇒ To learn how *T. cruzi* and *T. rangeli* evade the normal insect defence mechanisms and colonize the host.

⇒ To discover vector molecules responsible for transformation of the parasites from one stage to another.

⇒ Eventually to show whether compounds which induce immune-depression in the vectors can facilitate the control of Chagas' disease transmission.

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Contract number ERBTS3*CT930240

TRYPANOCIDAL DRUGS: LABORATORY AND FIELD EVALUATION OF NOVEL CONTROLLED RELEASE SYSTEMS

Period: January 1, 1994 - December 31, 1996

Co-ordinator: INSTITUTE OF TROPICAL MEDICINE PRINCE LEOPOLD, DEPT. ANIMAL HEALTH, Antwerpen, Belgium (S. GEERTS)

Objectives

- The objective of this research programme is to develop novel controlled release systems of homidium bromide, isometamidium and diminazene aceturate in order to increase the prophylaxis period.

- Other features of the new formulations which will be examined in detail: minimal risk of resistance development, minimal toxicity to livestock, minimal residual drug concentrations in the carcasses and overall cost of the new devices.

Activities

* Three European and two African institutions will undertake this research work in a coordinated fashion.

* The development of the improved formulations will primarily be the responsibility of the University of Ghent (polymer division). Different biodegradable (polymer) controlled release systems for subdermal implantation will be developed.

* Pharmacokinetic studies using radiolabeled drugs and sensitive analytical techniques for the detection of circulating drug concentrations will be developed by the University of Glasgow.

* Laboratory testing of the new formulations using laboratory animals and ruminants will be carried out at the Institute of Tropical Medicine, Antwerp and at Glasgow. Both institutes will also examine the possible development of resistance. Evaluation under realistic field conditions and cost-benefit analyses of the use of these controlled release trypanocides will be carried out under different conditions of management and tsetse challenge in 2 African countries (Mali and Zambia).

* The project will offer opportunities for participating scientists to visit other institutes for training and for research work.

Contract number ERBTS3*CT930240

Expected outcome

It is expected to collect scientific data about the value of different sustained or controlled release devices, based on currently available trypanocides, for the chemoprophylaxis of animal trypanosomiasis: composition (type of polymer, drug load, etc.), pharmacokinetics, protection period under laboratory and field conditions, effect on resistance development. These data should allow a comparative evaluation of these novel formulations with the trypanocidal drugs, which are currently available on the market.

Results

Development of drug delivery devices and in vitro release studies

Different types of sustained release devices, containing mixtures of biodegradable polymers and isometamidium (ISMM) or ethidium were synthesized. The effect of different parameters (solvent used for coating, dexamethasone in coating, coating thickness, buffer concentration and ionic strength, coating polymers, etc.) on the *in vitro* release of the devices was studied. The studies concerned mainly poly-(D, L-lactide) and poly-caprolactone-co-L-lactide) or poly-lactone-co-D, L-lactide).

Laboratory testing of the sustained release devices

After preliminary trials in rabbits challenged with *Trypanosoma congolense*, poly(D, L-lactide) polymers were selected for further evaluation in cattle.

Under controlled conditions (monthly challenge with tsetse flies infected with *T. congolense* clone IL 1180) it was shown that subcutaneous implantation of isometamidium-SRD (at 0.5 mg/kg) extended the prophylactic period by a factor 3.2 in comparison with intramuscular (i.m.) injection of the same doses of the drug. Similarly the protection period provided by the implantation of an ethidium-SRD (at 1mg/kg) was 2.8 times longer than that obtained after i.m. injection of the drug at the same doses.

Analysis of the isometamidium concentration in the sera of the treated animals (using ELISA) showed that the drug remained present for at least 5 months at concentration between 0.4 and 0.8 hg/ml in the SRD-treated cattle, whereas in the i.m. treated group the isometamidium concentration dropped already below the detection limit (0.5 ng/ml) 85 days post treatment.

When breakthrough isolates derived from treated rabbits or cattle were compared with the parent stock or clone of *T. congolese*, no evidence could be found that drug resistance was developing faster in SRD-treated than in conventionally treated animals.

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Pharmacokinetic studies and trypanocidal drug ELISA's

The drug disposition and pharmacokinetic parameters of homidium bromide and isometamidium were studied in different groups of cattle, infected or not infected with *T. congolese*. Furthermore, various modifications to the trypanocidal drug ELISA procedures were investigated in order to improve their sensitivity and specificity using field samples. These modified ELISA's were then used to analyze the isometamidium and ethidium concentrations in the sera of the cattle involved in the experiments under controlled and field conditions. The detection limit of the ISMM-ELISA was approximately 0.5 ng/ml.

Field trials using sustained release devices

In order to evaluate the efficacy of poly (D, L-lactide)-isometamidium-SRD under field conditions, a trial was carried out on a ranch in Mali where a heavy tsetse challenge was present. Two groups of about 40 cattle were treated with isometamidium (1 mg/kg), either as SRD or as i.m. injection, and compared with a control group. Eight months after treatment the cumulative percentage of trypanosome infections was 28, 59 and 77%, respectively in the SRD-implanted, the i.m. treated and the control groups. Statistical analysis proved that the infection risk was more than two times lower in the SRD-implanted group than in the conventionally treated animals.

A similar field trial was carried out in Zambia using poly (D, L-lactide)-ethidium-SRD. In this experiment 4 groups of about 20 cattle were involved. They received respectively the ethidium-SRD, ethidium i.m., ISMM i.m. or no treatment at all (control). The cattle, which received the ethidium-SRD were longer protected (average of 107 days) than those, which were treated with the same dosis of ethidium i.m. (average: 75 days). The average protection period provided by ISMM i.m. was similar to that of the ethidium-SRD. It has to be mentioned, however, that - contrary to the trial in Mali - resistant trypanosome strains were present in the area, where the experiment was done.

Other trials are currently being conducted in Mali and Zambia in order to further evaluate the efficacy of ISMM - and ethidium-SRD under field conditions.

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Contract number ERBTS3*CT930246

CHARACTERIZATION OF A NEW THERAPEUTIC TARGET ON AFRICAN TRYPANOSOMES: THE GLUCOSE TRANSPORTER AND DEVELOPMENT OF A VACCINATION STRATEGY

Period: December 1, 1993 - November 30, 1996

Co-ordinator: UNIVERSITE DE BORDEAUX II, URA CNRS 1637, LABO IMM. BIOL. PARASITAIRE, Bordeaux, France (T. BALTZ)

Objectives

The African Trypanosomes escape the immune defences of their hosts by expressing variable antigens on their surface. This phenomenon of antigenic variation has stimulated our research on the non-variable proteins on the surface which may have functions vital to the parasites survival. We have therefore characterized the glucose transporters of different Trypanosomes with the aim of testing their capacity to vaccinate and also investigated the possibility of developing new trypanocidal molecules based on modifications of hexose.

Activities

We have characterized the genes encoding the glucose transporters of several different species of Trypanosome: *T. brucei* and *T. congolense* which express two distinct isoforms, one expressed at all stages of parasitic development (THT2 and TcoHT2) and the other expressed uniquely on the trypomastigote stage (THT1 and TcoHT1), *T. vivax* (TvHT1) and *T. cruzi* which expresses a single isoform at all stages of the parasites development.

Alignment of the sequences revealed strong similarities (60-80%). The areas of divergence were at the ends of N and C terminal regions as well as in the first potentially extracellular loop which is rich in cysteine. Unlike mammalian glucose transporters, there was no systematic glycosylation.

While sequencing the gene encoding the *T. brucei* glucose transporter (50 Kb Cosmid) several new genes were discovered including PI3K (Phosphatidyl inositol Kinase) a protein with a structure comprising a repeated 43 amino acid, proline-rich motif with a few copies to about fifty copies depending on the isolate and the species and which constitutes a potentially useful epidemiological marker. "Run on" experiments have allowed us to identify a promoter. This would suggest that these genes are under the control of a promoter situated upstream. Studies of the organization of this region of the genome reveal strong similarities among the species.

The study of THT gene polymorphism in the different strains of the brucei group and in particular on *T. gambiense* has shown that there is great variation in the number of gene copies in *T. brucei* whereas variation in *T. gambiense* is low, this finding favors a clonal origin.

Direct functional studies on the glucose transporters of *T. brucei*, *T. vivax* and *T. cruzi* and following the expression of their genes in CHO have allowed us to draw the following conclusions.

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* All of the transporters are functional and are capable of transporting fructose unlike human glucose transporters and in particular Glut 1.
* High affinity (THT2 = 68μM, TcrHT1 = 84μM) and low affinity (ThT1 = 0.5 mM, TvHT1 = 0.58mM) transporters can be identified.
* Studies of substrate preferences have shown several important differences in the transporters THT1 is expressed on the blood stage forms of *T. brucei* and recognizes only derivatives of glucose with substitutions at position C6. This property has been exploited during our collaboration with the production of fructose with substitutions at position C6. This molecule (BrCH2 C(O)NH Fructose) is trypanocidal *in vitro* but without effect *in vivo.* Others strongly inhibit fructose transport but have no effect on parasites in culture.

We have tried to express the native THT1 protein in sufficient quantities for vaccination experiments in *E. coli* and *Baculovirus*, but without success. This may be explained partly by the difficulties in expressing any membrane protein. We have prepared a polyclonal rabbit antiserum against a twenty amino acid synthetic peptide representing the extreme C and N termini and against a fusion protein derived from the extracellular region which is rich in cysteines. Both antisera are trypanocidal when cultured in the presence of complement but neither prevents infection.

Conclusion

This study of the biochemistry and genetics of glucose transporters has allowed us to demonstrate the possibility of developing novel trypanocidal molecules with modified hexose bases. However, it has not been possible to express the transporter THT1 in sufficient quantities to permit an assessment of its capacity as a vaccine.

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Contract number ERBTS3*CT940266

CHARACTERIZATION OF THE IMMUNE RESPONSE AGAINST TRYPANOSOMA CRUZI ANTIGENS (GP 50/55 AND URINARY ANTIGEN) INVOLVED IN IMMUNOPATHOLOGY AND THEIR POTENTIAL USE IN DIAGNOSTICS

Period: June 1, 1993 - May 31, 1996

Co-ordinator: UNIVERSIDAD AUTONOMA DE MADRID, CENTRO DE BIOLOGIA MOLECULAR, Madrid, Spain (M. FRESNO)

Objectives

- To improve the understanding of the immune responses involved in protection and pathology in Chagas' disease in order to help control it.
- To characterize two antigens that may be involved in pathology and also may be good candidates for diagnostic.
- To study the cellular immune response against both antigens.
- To study the potential use of these two antigens as diagnostic tools.

Activities

* The functional and biochemical characterization of the *T. cruzi* antigen GP 50/55 which shares an epitope with a lymphocyte activation antigen and induces crossreactive antibodies in Chagasic patients which suppress lymphoid activity.
* The functional and biochemical characterization of a *T. cruzi* 80 kDa urinary antigen (UA).
* The cloning of the genes coding for the GP 50/55 and the UA proteins.
* To test the reactivity of chagasic sera from patients with different clinical status, with purified natural or recombinant GP 50/55 and the UA proteins.
* To test the reactivity of chagasic sera from patients with different clinical status, with purified natural or recombinant GP 50/55 and its possible relationship to differential diagnostic.
* To study the cellular immune response to *T. cruzi* and the role *in vivo* and *in vitro* of several cytokines. A special interest will be devoted to the study of the humoral and cellular response against the GP 50/55 protein and its role in pathology.
* To develop simple and highly sensitive methods for detection of parasite circulating antigens in urine to improve Chagas' disease diagnosis and follow-up of treated patients.

Results

We have investigated the biochemical and functional properties of *T. cruzi* GP50/55, a glycosyl-phosphatidylinositol (GPI)-anchored membrane antigen. Some of the properties (e.g. molecular mass, susceptibility to degradation) were reminiscent of those displayed by the *T. cruzi* lysosomal cysteine proteinase (GP57/51). A 50-52 kDa proteinase activity, specifically inhibited by thiol protease inhibitors, was immunoprecipitated with monoclonal antibodies (mAb) to GP50/55 (mAb C10), migrating slightly faster than the enzyme precipitated by mAbs to GP57/51. Moreover, the GP50/55 antigen detected by mAb C10 is expressed in the parasite membrane whereas the GP57/51 is not. The protein GP50/55 has been purified to homogeneity. We have found that the cystein protease activity copurifies with the GP50/55 protein (defined by reactivity with our monoclonal antibodies). However, the cysteine protease may be in fact a protein very tightly bound to GP50/55.

This has led to the identification of a mucin-like protein complex of 30,40 and 50kDa (AgC10) as the one recognized by mAb C10. The aminoacid composition and the structure of sugar chains have been elucidated. The epitope recognized by Mab C10 has been defined as well.

This purified protein is able to suppress the immune response against *T. cruzi* and selectively alters the production of tumor necrosis factor (TNF) but not interlukin - 1 (IL-1) by macrophages. These strategies may contribute significantly to the survival of the parasite.

On the other hand, the amino acid sequence of the N-terminal portion of an 80-kDa *Trypanosoma cruzi* urinary antigen (UAg) affinity - purified from the urine of infected dogs showed high degree of homology with human and dog transferrins. Whereas polycolonal antibodies were unable to discriminate between the parasite antigen and transferrin, some MAbs specifically and selectively recognized an 80kDa UAg but not host transferrin, and also reacted against a *T. cruzi* lysate.

Immunoprecipitation analysis showed that UAg specific antibodies bind to several trypanosome antigens including an 80 kDa polypeptide co-migrating with UAg. This UAg is a form of the host transferrin taken up and modified by the parasite. The nature of this modification is under investigation.

In agreement with those results it was not a surprise that we were unable to isolate the cDNA clone for a transferrin related UAg, by immunoscreening with polyclonal antibodies to transferrin and by PCR with degenerate oligonucleotides of conserved regions of transferrins. However, we succeeded to isolate a series of clones expressing the C-terminus portion of the tubulin protein of *T. cruzi*. This finding was also supported by immunoprecipitation experiments showing that the anti-UAg antibodies referred above (used for screening of library) were capable of recognizing tubulin. Simultaneously, we have determined the existence of a 50-55 kDa tubulin as a minor component of the purified UAg preparation, therefore secreted in the urine of infected dogs.

This recombinant antigen can therefore be used in the development of urine tests for diagnosis.

We have found that 100% of all human chronic chagasic sera reacts with this AgC10 complex which underlines the importance of this antigen as a potential candidate for diagnostics. Moreover, we have found that AgC10 induces cross-reactive antibodies that react with a 70kDa protein of lymphocytes. By screening of a human T-cell cDNA library with human chagasic sera, we isolated a couple of cDNA clones. One of those human clones (Cha 9.1.2) have homology with the repetitive region of the *T. cruzi* antigen SAPA, thought to be involved in the evasion of immune response.

This clone is recognized by a large percent of chagasic sera having cardiomyopathy. We have mapped to this site the reactivity of all autoantibodies in the chagasic sera.

Those results further expand our previous work, indicating that sera from chagasic patients recognize antigens present in human T and B lymphocytes. Moreover, the characterization of autoantibodies against lymphocytes may lead to the definition of a prognostic antigen for predicting the outcome of the disease.

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Presentation of EC supported joint research projects (1991-1996) continued
STD3
INCO-DC: 1st and 2nd Call

Areas of interest:

4. Leishmaniasis

Contract number: ERBTS3.CT920052

SYNTHETIC PEPTIDE ANTIGENS AS A TOOL FOR SPECIES-SPECIFIC SERODIAGNOSIS OF LEISHMANIASIS WITH FIELD APPLICATIONS IN BRAZIL AND COLOMBIA

Period: October 1, 1992 - September 30, 1995

Co-ordinator: LIVERPOOL SCHOOL OF TROPICAL MEDICINE
Liverpool, United Kingdom (M. HOMMEL)

Objectives

The overall objective is to evaluate a series of synthetic peptides, derived from *Leishmania* genes, which can be used, under field conditions, for species-specific serodiagnosis of leishmaniasis. The original rational behind the proposed study was the finding that a synthetic 15 amino-acid peptide, based on the sequence of a *Leishmania donovani* gene, could be used in the laboratory for a specific serodiagnostic test of visceral leishmaniasis.

The main objectives of the study are:

- To field-test the existing peptide assay in selected areas of Brazil and Colombia, endemic for visceral leishmaniasis in order to evaluate its predictive value.
- To perform similar studies in areas for (muco-)cutaneous leishmaniasis and Chagas' disease in order to confirm species-specificity.
- To further improve the peptide-carrier construct.
- To use the same rationale and technology to be applied to the *Leishmania braziliensis* complex.
- To include peptide-carrier constructs in assays suitable for use under field conditions.
- To set up a laboratory of molecular biology at the University of Sucre.
- To collect isolates of Leishmania and patient blood and sera from locations in Colombia and Brazil.

Activities

* Improvement of the methodology for the preparation of peptide-carrier constructs by conjugation of peptides to the human serum albumin carrier using thi-oester hetero-bifunctional reagents.
* Design of new mixtures of peptide sequences ("mixotopes") based on consensus and variable motifs of the leishmanial rK39 sequence, an antigen which has proven diagnostic value.
* Testing the potential use of random peptide phage display libraries as a means for the identification of immunodominant leishmanial peptides.
* Investigation of the scientific basis for the direct agglutination test using whole leishmanial promastigotes.
* Collection and PCR characterization of leishmanial isolates from Colombia.
* Development of an antigen-capture assay for the detection of leishmanial antigens in the urine of patients with visceral leishmaniasis.
* Collection of serum from patients with clinical visceral leishmaniasis, cutaneous and mucocutaneous leishmaniasis, Chagas' disease, asymptomatic leishmanial infections and a variety of endemic controls in Brazil.

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Results

⇒ Development of a new, ELISA-based, serological assay for the diagnosis of visceral leishmaniasis using a mixture of synthetic peptides linked to a carrier protein (HSA). Publication of the description of the assay and its performance in scientific literature.

⇒ Characterization of new foci of leishmaniasis in Brazil and in colombia (District of Sucre).

⇒ Completion of 3 PhD theses on leishmaniasis (1 from a DC student, 2 from EU students); 2 other PhD theses from DC students are still in the process of completion.

⇒ Setting up of a laboratory for the study of leishmaniasis and the use of molecular biology methods in Sincelejo, Sucre, Colombia. This laboratory has been given the status of Regional Leishmaniasis Reference Laboratory by the Colombian Health Authority.

⇒ Organization in Liverpool of one of the Euroleish Network meetings and editing of the proceedings.

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Contract number ERBTS3*CT920113

VISCERAL LEISHMANIASIS: EPIDEMIOLOGY AND DISEASE CONTROL

Period: September 1, 1992 - August 31, 1995

Co-ordinator: LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE
London, United Kingdom (M.A. MILES)

Objectives

The following are the main objectives:

- To establish research facilities at the University of Teresina and a training programme for local staff.
- To perform a thorough comparison of diagnostic serology and diagnostic parasitology for Brazilian and European canine and human visceral leishmaniasis, incorporating innovative diagnostic procedures.
- To assess aminosidine (paromycin) for the treatment of canine visceral leishmaniasis.
- To identify asymptomatic dogs and people and to determine whether such carriers can act as a reservoir of infection.
- To recommend and implement improved strategies for disease control based on research findings from the programme.

Activities

* Comprehensive epidemiological data will be assembled from the records held at the Ministry of Health and the University at Teresina. A thorough comparison of the latest appropriate technologies for the diagnosis of canine VL will be undertaken with a minimum sample size of 100 dogs assembled at the Centre for Zoonoses.
* A colony of *Lutzomyia longipalpis* will be established at the University of Teresina; flies will be infected by feeding on dogs with heavy skin infections of *L. chagasi* and the infections will be transmitted to an experimental group of animal imported from a non-endemic area.

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* The chemiluminescent probe will be tested for its ability to detect *L. donovani* in experimentally infected and wild-caught *Lu. longipalpis*, and compared with detection by microscopy. A prototype diagnostic field kit will be assembled.

* Using dogs with moderately severe VL (without severe wasting) the efficacy of aminosidine treatment, using various regimes, will be investigated. The infection will be monitored using sequential bone marrow and skin biopsy techniques.

* Improved serological techniques will indicate potential asymptomatic human carriers of VL and detailed epidemiological information on suburban visceral leishmaniasis will be available as a result of this study. This epidemiological analysis will be used to recommend improved methods for disease control.

Expected outcome

The following outcomes are expected from this project:

⇒ A detailed epidemiological description of visceral leishmaniasis will become available as a result of this project.

⇒ The chemiluminescent DNA Probe will be evaluated in the field, and aminosidine trials will have been conducted in dogs.

⇒ Conclusions be drawn on the transmissibility of the disease from asymptomatic carriers.

⇒ Recommendations will be made on the best diagnostic procedures that are currently available and on improved strategies for disease control.

Results

⇒ Data assembled include: incidence of human VL (1981 - 1994); incidence of human VL by age and by sex; suburban distribution of human VL.; suburban distribution of canine seropositivity; records of suburban and periurban sandfly species; suburban distribution of insectivide spraying.

⇒ Clinical, parasitological and serological diagnostic methods have been compared with a cohort of more than 200 naturally infected dogs, and an *L. donovani*-complex specific DNA probe assessed. A DNA-based diagnostic kit was described. An *L. donovani* complex specific colorimetric (visual) PCR assay was developed.

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⇒ The *L. donovani*-complex specific probe was shown to be an effective tool for detecting *L. chagasi* infections in wild caught sandflies.

⇒ *L. chagasi* was highly transmissible from dog to dog by *Lutzomyia longipalpis*, infectivity to sandflies was compared with clinical status.

⇒ A cohort of dogs infected experimentally by sandfly bite was established, and parasitological positivity, serological conversion and transmissibility of infection followed.

⇒ A trial of aminosidine for treatment of canine VL was performed: clinical recovery, limited cure, and some adverse effects were obtained.

⇒ A combination of serology and a gamma interferon capture assay detected putative asymptomatic VL among families with index clinical cases. Carrier status is under investigation (in collaboration with Dr. Carlos Henrique Costa). Distribution of selected human genotypic markers within the study cohort have been determined (Oxford, UK).

Results on the diagnosis and transmissibility of canine VL question the efficacy of serological surveys and killing of dogs as efficient means of VL control.

Contract number ERBTS3*CT920113

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Contract number: ERBTS3*CT920123

RECOMBINANT ANTIGENS AS SEROLOGICAL TOOLS FOR SPECIFIC AND SENSITIVE TERGUMENTARY AND VISCERAL LEISHMANIASIS DIAGNOSIS

Period: April 1, 1993 - March 31, 1996

Co-ordinator: UNIVERSIDAD PERUANA CAYETANO HEREDIA
Lima, Peru (Y. MONTOYA)

Objectives

To develop rapid, sensitive and highly specific tests based upon recombinant peptide antigens for the improved diagnosis of Visceral leishmaniasis (VL) and American tegumentary leishmaniasis (ATL)

Activities

- Sera from patients co-infected with VL and HIV were followed up by Western blot using *L.(L) infantum* total proteins as antigens and by ELISA using rK-39 recombinant protein.
- Construction and screening of *L.(L.) infantum* cDNA libraries using sera from patients co-infected with VL/HIV.
- Selection and characterisation of the candidate recombinant antigens for *L.(L.) infantum* and *L.(V.) peruviana*.
- Assessment of the diagnostic potential of two candidate *L.(V.) peruviana* recombinant clones.
- DNA sequencing of the two selected *L.(V.) peruviana* recombinant antigens, their derived synthetic peptides and the parasite extract as diagnostic reagents for ATL and VL were compared.
- Identification and selection of highly specific and sensitive *L.(V.) peruviana* epitopes by using synthetic peptides.

Outcome

⇒ The development of an improved sero-diagnostic test in terms of greater specificity, sensitivity and predictive value over conventional tests using recombinant antigens.

⇒ One PhD, one MSc and four Licentiate of Biology thesis from Peruvian students were finished as part of this project. Likewise, one PhD thesis from Spain will be obtained.

Results

Research in the last three years identified two extremely promising *L.(V.) peruviana* recombinant proteins referred as "T26-U2 plus T26-U4" which were recognised by individual sera from ATL patients with successful results. The combined sensitivity=87%, combined specificity=87% and the combined total predictive value=87% obtained is a good alternative to diagnosis.

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The regional coverage was assessed with eleven Colombian sera and the sensitivity found was 92% suggesting that "T26-U2 plus T26-U4" could be used successfully in other Latin American countries.

"T26-U2 plus T26-U4" recombinant clones were DNA sequenced and their predicted aminoacid structure allowed us to design *L.(V.) peruviana* synthetic peptides containing B-cell epitopes. The use of these synthetic peptides improved the specificity (92%) and the sensitivity was comparable with the obtained with "T26-U2 plus T26-U4" recombinant protein. Furthermore, we included a small panel of sera of VL patients from Spain and these sera reacted with the *L.(L.) infantum* total proteins by Western blots. Variable patterns of antigenic bands were found during comparison among sera from patients that were apparently cured and those who had relapsed.

Likewise, *L.(L.) infantum* cDNA libraries were prepared and after screening with a pool of sera from VL patients co-infected with HIV, identified fifteen *L. (L.) infantum* recombinant cDNA clones. Their expression products were assessed with individual sera of VL/HIV patients with satisfactory results.

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Contract number: ERBTS3*CT920129

CLONAL VARIABILITY OF THE PARASITE AS A PREDICTIVE TOOL FOR DIFFERENT CLINICAL MANIFESTATIONS IN TEGUMENTARY LEISHMANIASIS OF PERU AND BOLIVIA

Period: October 1, 1992 - September 30, 1996

Co-ordinator: INSTITUT VOOR TROPISCHE GENEESKUNDE "PRINS LEOPOLD", LAB. OF PROTOZOOLOGY
Anvers, Belgium (D. LE RAY)

Objectives

Identification of molecular marker(s) that correlate(s) with mucosal compromise of New World Tegumentary Leishmaniasis. Afterwards, they may be used to assess its or their predictive value.

- To follow-up approximately 100-150 patients in Peru and Bolivia from whom isolates will be obtained for subsequent molecular analysis.
- To develop new DNA oligonucleotides or specific probes to improve DNA heterogeneity analysis from the population genetics point of view.
- To build up clustering patterns of Leishmania isolates by the use of phenotypic and genotypic markers.

Activities

Field work studies:

* Patients selection: patients will qualify for the study if they have active cutaneous or mucocutaneous lesion with demonstration of parasite without previous specific therapy, and if they attest their willingness to participate in the study.
* Patients follow up: after conventional treatment with antimonial pentavalents patients will be followed up for any evidence of mucosal compromise. The patients however will become aware that they should visit the health post at the first sign of mucosal distress or cutaneous lesion relapse.
* Parasite isolation: by cultivation of biopsy or aspirate samples from patients with cutaneous and/or mucosal lesions before chemotherapy, and from cutaneous, treated patients who will develop mucosal compromise during follow-up.

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Laboratory work to include:

* Isoenzyme analysis

* Random Primer Amplification Method (RAPD)

* Schizodeme analysis

* Karyotype analysis of Leishmania by PFG

Data analysis:

* Polymorphism obtained through the methodologies described above will be analyzed in terms of population and evolutionary genetics.

Expected outcome

* To obtain clustering patterns after characterization of Leishmania isolates that can answer the question whether genetic markers can be correlated with clinical categories of the disease.

* To obtain new DNA oligonucleotides or probes specific for parasites causing tegumentary leishmaniasis. As PCR will be tried for diagnosis under field work conditions, we expect to simplify this methodology.

Results

Field activities have framed the endemic survey area in Bolivia (Ivirgarzama Health District, Cochabamba Dpt) while in Peru a field laboratory was erected at the Pilocopata Health Post (Madre de Dios, Cuzco) and equipped for *in vitro* cultivation, PCR tests and PC data processing. One hundred and sixty seven Leishmania parasites, all with well documented clinical records, were collected: seventy-nine from Bolivia and eighty-eight from Peru. More isolates were obtained from cutaneous lesions than from mucosal ones (128 vs 39 respectively). Part of them (40) has been subjected to molecular analysis.

For molecular karyotype studies (Belgium), several DNA probes, each recognizing a different chromosome, were tested for polymorphic karyotype patterns. Six new probes were isolated from a cosmid library of *L. braziliensis* (courtesy of D.G. Barker, Cambridge, UK), they are candidate probes for future studies on karyotype polymorphism of sympatric isolates from the study areas.

From a phenetic analysis of 4 sets of chromosomes in *Leishmania peruviana* and *L. braziliensis,* concordance between geographical and genetic patterns was evidenced and a North-South populational gradient was identified for *L. peruviana* along the Peruvian Andes. Northern *L. peruviana* were closer to *L. braziliensis.* Specific chromosomal size-differences were suggestive of chromosome rearrangement and their possible association with specific clinical forms was identified as compatible with the working hypothesis subtending the global project.

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Nucleotide sequence of the chromosomal probe pLb-134p corresponded to the gp63 gene, whose product has been associated with Leishmania virulence by other authors. Quantitative and qualitative variation of gp63 genes was evidenced among Leishmania species of the *L. braziliensis* complex. This conclusion was established after RFLP analysis. The particular organization of gp63 genes led to the development of a PCR-based characterization method. Primers to amplify the most informative region of the gp63 genes have been obtained, and evaluation is in progress.

With the chromosomal probe pLb-22, the nature and organization of ribosomal genes in discrete clusters along corresponding chromosome were identified. Primers for DNA amplification of ribosomal DNA were developed. They amplified either the intergenic sequence (IGS) or the internal transcribed sequences (ITS). Both presented polymorphic traits in the studied isolates.

In an outbreak in the Eastern Andean valleys, both *L. braziliensis* (sylvatic cutaneo-mucosal leishmaniasis) and *L. peruviana* (Andean cutaneous leishmaniasis) were isolated in sympatry and, putative hybrids were found.

The Montpelier group (France) proceeded to multi-locus enzyme analysis. (MLEE, 16 polymorphic loci) of 30 isolates from Peru, Bolivia and Ecuador. RAPD (with 6 different primers) was evaluated on 50 isolates in parallel to MLEE. Beside discrimination of *L. peruviana* from *L. braziliensis, L. guyanensis and L. lainsoni* were detected in Peru for the first time. Genetic tests showed that leishmanial Andean populations along the North-South transect through Peru displayed a geographical structure as well as a strong departure from *panmixia* in the central area, suggestive of clonality. There was a highly significant correlation between MLEE and RAPD genetic distances and - in most instances but for some specific locations - both MLEE and RAPD did corroborate karyotype data.

Preliminary results showed a strong linkage disequilibrium of RAPD data in natural populations. Contradiction with karyotype data was observed and deserves further attention. Clonality appears to be the principal reproduction mode in Leishmania, but pseudo-sexual phenomena might be present.

Respective prevalences of different genetics markers in parasites isolated from mucosal and cutaneous patients support our initial hypothesis that there is a genetic basis for virulence.

Contract number: ERBTS3*CT920129

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Contract number ERBTS3*CT930245

VISCERAL LEISHMANIASIS IN THE SUDAN: AN EPIDEMIOLOGICAL AND ENTOMOLOGICAL STUDY

Period: January 1, 1994 - December 31, 1996

Co-ordinator: UNIVERSITY OF AMSTERDAM, ACADEMIC MEDICAL CENTRE, Amsterdam, The Netherlands (P. KAGER)

Objectives

Epidemiology

- To study the epidemiology in an endemic area of VL in eastern Sudan, including the prevalence of clinical and subclinical cases and complications as Post-Kala-Azar Dermal Leishmaniasis.
- To evaluate the performance of the Direct Agglutination Test under field conditions in the Sudan.
- To evaluate the performance of new molecular biological tools in the diagnosis of VL and during follow-up.

Entomology

- To determine the population dynamics of the vector and thus the time of the year when the transmission of VL takes place. To describe the circadian cycle of the vector to show the time of maximum risk.
- To investigate the main resting sites of the sandfly vector.
- To study the host preferences of the vector to indicate putative animal reservoirs of infection.

Activities

The Sudan has by far the most important leishmaniasis problem in Africa. A major epidemic is taking place in south Sudan, since 1988, causing thousands of deaths. The clinical course of infection and epidemiology of Sudanese VL are imperfectly known. This proposal addresses the following questions:

Epidemiology

Sero-epidemiological surveys will be carried out during which the prevalence of clinical and subclinical cases and Post-Kala-Azar Dermal leishmaniasis will be assessed. Some of the individuals have been previously exposed to *L. major*. The value of the Direct Agglutination Test will be evaluated under field conditions. In addition, new molecular biological techniques such as PCR and *in situ* hybridization will be tested in diagnosis, after treatment as test of cure and as predictors of possible relapse. This will be done on aspirates of lymph node, bone marrow and spleen and on peripheral blood.

Entomology

Population dynamics of the vector as well as main resting sites will be determined. Host preferences of the vector to indicate putative animal reservoirs will be studied.
Several components of training and transfer of technology are included in the proposal. The results may lead to the design of a control programme.

Results so far

Epidemiology

Yearly 2 surveys of the total population of 2 villages, Um Salala and Mushra Koka, were done. The DAT proved a useful and reliable tool, the freeze antigen proved to be an advantage.

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This test does not differentiate between active or past infection however and can not be used as "test of cure". Within a certain epidemiological and clinical context, the DAT may replace invasive diagnostic procedures. Lymph node aspiration is a reliable diagnostic procedure for VL with few complications.

PCR on lymph node material proved very valuable, in peripheral blood the PCR was positive in about 79% of VL patients. In PKDL patients PCR was a useful method both on materials from the skin and from lymph nodes. Whether PCR (in relation to LST reaction and DAT, or not) can be helpful in prediction relapse, development of PKDL or cure is bering analyzed. Laboratory work and analyses are ongoing.

Entomology

Field studies were done in and around villages and in adjacent Dinder National Park where visceral leishmaniasis occurs in the game wardens. Very few sandflies were caught in the village, 100 times more in the forest in the park. *P. orientalis*, a proved vector, was caught in termite hills. A colony of P. orientalis was established, but unfortunately lost when the team was working in the field.

Preliminary studies of host preference of *P. orientalis* did not indicate a preference of *Arvicanthis niloticus*, a common rodent that could possibly be a reservoir animal (in the park a zoonotic situation is suggested, in and around the villages man to man transmission is likely).

The biology of *P. orientalis* in this area of Sudan appears to differ from the situation in Upper Nile Province, where intensive studies of this sandfly were performed some thirty years ago.

Work and analysis are ongoing.

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Contract number ERBTS3*CT930247

MOLECULAR TECHNIQUES FOR VECTOR AND PARASITE IDENTIFICATION APPLIED TO A PILOT VECTOR CONTROL STUDY OF LEISHMANIASIS

Period: January 1, 1994 - December 31, 1996

Co-ordinator: UNIVERSITY OF KEELE, DEPARTMENT OF BIOLOGICAL SCIENCES, Staffordshire, United Kingdom (R.D.C. MAINGON)

Objectives

Leishmaniasis, a rural and periurban disease of high morbidity and prevalence in tropical areas and in the Mediterranean, is transmitted by a variety of sandfly species. Generally, due to outdoor transmission, vector control is of limited value in diminishing the incidence of leishmaniasis except in those instances where transmission is predominantly inside houses.

The overall goal is to use previously established molecular techniques to investigate the potential preventive value of permethrin-impregnated curtains from reducing manbiting rates in a pilot control study in a selected highly endemic focus of cutaneous leishmaniasis. The present study is a pre-requisite for further intervention vector control trials aimed at reducing the incidence of the disease in areas of domestic transmission.

Specific objectives

- To determine the vectorial capacity of anthropophilic sandfly species in two ecologically different regions (Lara and Miranda states).
- To gather epidemiological information in the Guayamure/Rio Claro of Lara and Miranda state using molecular techniques combined with classical field methods.
- To evaluate the efficacy of permethrin-impregnated curtains against endophilic phlebotomine sandflies with respect to a reduction of biting rates in a highly endemic pilot area in Lara state and Miranda states.

Activities

Extensive field work indicated a high level of endophilic transmission of cutaneous disease in El Ingenio, Miranda State and to a lesser extent in Guayamure and Rio Claro villages in Lara State, Venezuela. El Ingenio village consists of 54 houses with a population of 254 inhabitants of predominant agricultural occupation.

This and other features such as high prevalence of cutaneous cases, long term surveillance of these villages made them suitable to investigate the efficacy of insecticide-impregnated curtains in reducing intra-domiciliary sandfly number and their biting rates.

This project is in its third year. During the first year of the project the entomological analysis of the densities and sandfly species inside vs outside house in El Ingenio provided the base line data for selecting the study and control houses for a pre-pilot vector control trial. This aimed to gain an insight into the relative value of all variables involved in an effective trial. This preliminary trial took place at the end of the first year between November 1994 and April 1995. Deltamethrin at a dose of 15 mg/m^2 was used to soak curtains (6mm mesh size).

During the second year of the project, a number of issues related to the El-Ingenio trial have been examined to identify specifically the cause(s) for the apparent lack of vector control:

* Sandfly trapping methods used for monitoring the trial.

* Variables affecting sandfly susceptibility to a number of insecticides such as insecticide source and concentration and curtain fabrics and curtain mesh size.

* Changes in the local sandfly population density and/or sandfly behavior.

* Changes in the community behavior particularly of those people living in the selected houses.

Results

Progress towards the specific objectives of the original proposal is summarized below:

⇒ The vectorial capacity of anthropophilic sandflies in El Ingenio and Altragracia de Orituco (Northcentral Venezuela) has been partially elucidated. Since dissection to find out natural infection rates is currently being carried out, it will be important to implement the PCR technique with pooled sandflies (of a given species, i.e. ovallesi with Le. braziliensis specific primers, MpL 1 and MP3H).

⇒ Epidemiological information in the Rio Claro and Guayamure foci of Lara and in Altragracia de Orituco (Guarico State) and El Ingenio (Miranda State) has used classical and molecular techniques except in the Northern region. Implementation of human DNA detection by PCR in sandfly bloodmeals would enhance the sensitivity of ELISA detection.

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For evaluating the efficacy of permethrin-impregnated materials against endophillic sandflies a second three-way trial is required comparing both vector control methods: insecticide-impregnated curtains with insecticide wall spraying inside houses in El-Ingenio. Due to weather pattern changes there has been very reduced number of sandflies in both places throughout 1995. The three-way trial is scheduled to take place as soon as the sandfly density increases.

An intensive course in molecular entomology organized by Dr. Maingon in Maracay during April 22 - May 6 funded by WHO-PAHO was very well received by the Latin American students on the Maracay based M Ses on Medical Entomology. Agricultural Pests and Public Health. The course consisted of 10 lectures of 2h each followed by practicals 5h each. Topics comprised DNA probes, species-specific PCR, RAPD-PCR, insecticide resistance mechanisms, immunity in insects, the molecular biology of parasite-insect interactions. A manual in Spanish of Protocols in Molecular Entomology was produced. Also in Spanish, a practical manual on sampling and mounting sandflies has been produced for field workers by Drs. M. Maroli and Feliciangeli. Two PhD studentships (one British and one Venezuelan) are sponsored by this project.

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Contract number ERBTS3*CT930253

COMPARATIVE EVALUATION OF CLASSICAL AND MOLECULAR TOOLS FOR THE DIAGNOSIS AND FOR ECO-EPIDEMIOLOGICAL INVESTIGATIONS OF LEISHMANIASIS

Period: October 1, 1993 - September 30, 1996

Co-ordinator: INSTITUT PASTEUR DE TUNIS
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Objectives

- To develop a DNA probe specific for *Leishmania infantum*; to measure its general efficiency for diagnosis of human and canine leishmaniasis (in particular during disease development in the dog), using the dot blot technique; to evaluate the results with those obtained by non-molecular, classical methods.
- o evaluate the comparative sensitivities and specificities of kDNA probes for the identification of *L. infantum.*
- To measure cytokine production by PBMC in response to parasite antigen to understand the TH1 versus TH2 balance in clinical groups as a function of age.
- To measure the intrinsic validity and extrinsic validity parameters for DNA probes specific for *Leishmania major*, as used in the dot blot technique for the diagnosis of zoonotic cutaneous leishmaniasis in man; and, to compare the results with the estimates of these parameters for classical techniques (direct examination, culture, inoculation of animals, serology)
- To evaluate squash blotting, ELISA and classical dissection techniques for the detection of Leishmania in the phlebotomine vector, for the pairs *Phlebotomus papatasi/ Leishmania major, P. perniciosus/Leishmania infantum and P. perfiliewi/L. infantum.*

Activities

Phlebotomine sandflies necessary for the various molecular tasks were successfully collected from different regions of Tunisia.

For the evaluation of the sensitivity and specificity of probes for dot-blotted promastigotes of *L. infantum* 3E9/HaeIII-12 and 3B8/HaeIII-2, the two mini-circle kinetoplast (k) DNA probes were tested using 49 promastigote preparations, including 7 different species of Leishmania and *Sauroleishmania tarentolae* originating from several Old World countries. Promastigotes were cultured in RPMI medium with foetal calf serum. Serial dilutions of 10^6, 10^5, 10^4 and 10^3 promastigotes were applied to replicate nylon DNA transfer membranes using a vacuum blot apparatus.

For the evaluation of the diagnostic potential of probes specific for *L. infantum* infecting man and dog and comparison with classical techniques samples were collected from 32 human cases and 152 dogs.

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In a basic health care centre in the Sidi Bouzid focus, 54 patients were selected as having ZCL on a clinical basis (epidemiological context, clinical presentation and site of lesion, duration of lesion for more than 3 weeks, inefficacy of antibiotics). The patient sample constituted 25 males (43%) and 29 females (57%) varying in age from 2 to 81 years old. The number of lesions per patient varied from 1 to 11 (average = 3). 30 patients had already started a course of Glucantime treatment.

Samples were taken from each lesion for

* A direct dermal smear on a microscope slide was colored by the May-Gunwald-Giemsa technique and read with an optic microscope at x1000 magnification (54 patients).
* A culture was made in NNN medium with Penicillin G (800 U/ml rabbit blood and Streptomycin (500mg/ml rabbit blood) (49 patients).
* 3 to 6 drops of dermal fluid (ca. 5ml each) were spotted on to a Genescreen Plus nylon DNA transfer membrane; after the normal denaturation and neutralization processes, each membrane was treated with proteinase K (100 mg/ml in 0.1M Tris-HCl pH 7.5) for 1 hour at 37°C (54 patients).
* Dermal fluid was inoculated in to the hind footpads of a Balb/c mouse.
* A blood sample was taken for ELISA and IFAT tests.

Results

In general, serological tests were the most sensitive (73% for IFAT, 94.6%% for ELISA), followed by the Balb/c inoculation (48.2%).

⇒ Balb/c mice (MI): the lesions appeared from 4 to 9 weeks (max 67 days).

⇒ NNN cultures (C): 12 were positive within the first week and 3 in the second week of subcloning. Only one of the samples giving a positive culture was negative in Balb/c mice.

⇒ Direct smear (DS): of the 26 samples positive in Balb/c mice only 12 (46.15%) were also positive by direct smear, but 4 smears were positive when the Balb/c results were negative.

⇒ ELISA: soluble antigens were prepared by the classical technique from a Tunisian strain of L. major (MPSA/TN/86/RON44; zymodeme MON-25/LON-1) and uses at 1/500 dilution; sera were diluted at 1/100; positive and negative controls were used on each ELISA plate; spectrophotometry reading was at 420 nm.

⇒ IFAT: the same strain of L. major was used; sera were diluted 1/50 in PBS, and the fixed antibodies were revealed with anti-human antiglobulins labeled with fluorescein.

⇒ Dot blots: samples from 54 patients were hybridized with the 85 bp universal probe (SU), the 450 bp TaqI probe (TaqI) and the 250 bp AvaII probe (AvaII) (see section B.2.2 of first technical report). Autoradiographic exposure was set at one week for the first two probes and two weeks for the third probe. The presence of Leishmania parasites in the samples was confirmed by at least one direct test. The variable number of dot-blot samples (3-6) made for each lesion was shown to be adequate, even though the intensity of the autoradiographic signal varied both within and among lesion samples.

However, only some of the patients' lesions had demonstrable parasites, and so the evaluation of the various indirect techniques can be better estimated after selection of the positive patient sample. This "gold standard" was the positivity demonstrated by at least one of the three classical techniques that allow visualization of the parasite, namely direct smear, culture and Balb/c inoculation. The gold-standard sample is constituted by 30 patients among the total of 54. For this sample the dot blot sensitivity was 46.7% (14/30) or 40.0% (12/30) depending on the probe. The 24 patients not showing any parasites in their lesions, when assessed by the three gold-standard tests, constituted the negative control sample that permitted calculation of the specificity of each of the indirect tests. The specificity was 100% for all techniques except for ELISA and IFAT.

Probe 3E9/HaeIII-12 (= 3E9) can be considered as an excellent tool for the specific identification of *L. infantum* in Tunisia (and in the Mediterranean Basin in the absence *of L. donovani*) when 10^5 or fewer promastigotes are used: then, both the specificity and predictive value of the positive result were 100%. As with most diagnostic tools, there is a trade-off between specificity and sensitivity, with a loading of 105 promastigotes being optimal.

Probe 3B8/HaeIII-2 (=3B8) was assessed to be less efficient than 3E9, its sensitivity being one order less and the predictive value of a negative result never reaching 100%.

Achievements

Measurement of the intrinsic validity parameters for DNA probes specific for *L. major*, as used in the dot blot technique for the diagnosis of ZCL in man, and comparison of the results with the estimates of these parameters for classical techniques (direct examination, culture, inoculation of animals, serology).

Using the KDNA probes previously isolated, the dot-blot DNA test was shown to be 100% specific, as were the three direct visualization tests (direct smear, Balb/c mouse inoculation, NNN culture). The serological tests (IFAT, ELISA) showed significantly lower specificity but higher sensitivity. Inoculation of Balb/c mice proved to be the test with highest "global efficiency". The dot blot test is, therefore, a useful addition to the battery of tests now available for diagnosis of ZCL due to *L. major*, performing as well as all tests except inoculation of Balb/c mice and, unlike the serological tests used, having 100% predictive value of a positive result.

Confirmation was obtained of the diagnostic value of a ribosomal IGS DNA probe for the identification in different regions of Tunisia of *P. papatasi*, the only known vector of *L. major* in north Africa. Development was started of DNA probes specific for the most abundant and widespread Tunisian vectors of *L. infantum*: restriction mapping, subcloning and sequencing permitted the identification of DNA fragments and internal repeat sequences that showed marked specificity for *P. perniciosus* and *P. perfiliewi*.

Contract number ERBTS3*CT930253

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Contract number ERBTS3*CT940263

ANALYSIS OF PHOSPHOFRUCTOKINASE AND PYRUVATE KINASE OF LEISHMANIA, POTENTIAL TARGETS FOR NEW DRUGS

Period: January 1, 1994 - December 31, 1997

Co-ordinator: ICP, RESEARCH UNIT FOR TROPICAL DISEASES
Brussels, Belgium (P. MICHELS)

Objectives

To contribute to the development of new drugs against leishmaniasis, by a rational approach. Enzymes playing a key role in the metabolism of the parasite will be selected as targets for new drugs. The target enzymes of the parasite will be studied in very great detail, to search for structural and kinetic differences with the enzymes of the mammalian host. Selective inhibitors will be designed that exploit these differences. Such inhibitors might be used as lead compounds for drug development.

Activities

Glycolytic enzymes were chosen as targets for drugs to be designed, because glycolysis plays an essential role in the energy and carbohydrate metabolism of *Leishmania*. Phosphofructokinase (PFK) and pyruvate kinase (PYK), key enzymes of the glycolytic pathway, are promising candidates for selective inhibition, because the parasite's enzymes have some unique structural and regulatory properties. The genes will be characterized. Subsequently, the proteins will be overexpressed in *E. coli*, purified and used for detailed kinetic studies. The primary structure of *Leishmania* PFK and PYK will be modelled into a three-dimensional structure, using the available crystal structure of homologous proteins. Detailed information about enzyme-ligand interaction will be obtained by combining the results of the kinetic analysis, binding assays and molecular modelling. From this information hypotheses will be constructed as to how unique (sub)domains in the *Leishmania* proteins could be involved in specific kinetic features. The hypotheses will be tested by site-directed mutagenesis and subsequent analysis of the mutated proteins. Those domains conferring unique kinetic features to the proteins will be used for designing selective inhibitors that could be used as lead compounds for the development of chemotherapeutic agents.

In parallel with the above described studies, the bacterially-overproduced PFK and PYK will be used in crystallization trials. If good crystals can be obtained, they will provide us with the most accurate information about the three-dimensional structure of the enzymes.

Results

Phosphofructokinase

The initial analysis of PFK was performed using the enzyme of *Trypanosoma brucei*, an organism closely related to *Leishmania*, because the trypanosome enzyme was already available in purified form before the start of the project. Amino-acid sequences of some peptide fragments from the purified PFK were determined and used to select degenerate oligonucleotides, for amplification of a PFK gene segment by PCR. Subsequently, this amplified segment was used to identify clones containing PFK genes in genomic libraries of *T. brucei*, *L. mexicana*, *L. donovani* and *L. major*. Sequence determination of the *Leishmania* gene is in progress, whereas the *Trypanosome* PFK gene characterization has been completed.

The *Trypanosome* PFK appears to be very different from the human enzyme (20% amino-acid identity), reinforcing the notion that his enzyme is a promising target for drug

design. Very striking is the observation that this ATP-dependent PFK of the Trypanosomatidae is more related to the PPi-dependent PFKs of anaerobic protests and bacteria (up to 40% identity) than to any other ATP-dependent enzyme. The enzyme has now also been purified from *L. mexicana* promastigotes, and will be used for kinetic analysis.

Pyruvate kinase

The PYK gene of *L. mexicana* has been cloned and characterized. The encoded polypeptide has about 50% amino-acid sequence identity with its mammalian homologue. The *Leishmania* enzyme has been over-expressed in *Escherichia coli* and purified from the bacteria. The enzyme has also been purified from *L. mexicana* promastigotes. The bacterially expressed enzyme appeared to differ from the natural protein in two respects: the value of some kinetic parameters was significantly different and the isoelectric point of the two proteins have a value of 5.90 and 5.75, respectively. These differences are attributed to phosphorylation of the promastigote PYK. Such phosphorylation could play an important physiological role in the activity regulating of the enzyme. This is currently under investigation.

Considerable efforts have been made to crystallize the *Leishmania* PYK expressed in *E. coli*. Crystals have been obtained, but their size and quality remain to be improved before the enzyme's structure could be solved. Currently, mutants are being prepared in an attempt to produce a more rigid enzyme to aid its crystallization.

Based on the amino-acid sequence of both the *L. mexicana* PYK and the related *T. brucei* enzyme, models have been made of their three-dimensional structure, using the available X-ray coordinates of two mammalian muscle PYKs. In these models, a pocket could be identified possibly containing the binding site of fructose 2, 6-bisphosphate, a potent allosteric effector, specific for the trypanosome. This site is a potential target for selective drugs and is being studied further by site-directed mutagenesis. Indeed, some mutants have been obtained in which the affinity for fructose bisphosphates, and their effect on the activity of the enzyme was affected. Additional mutants are being made to obtain further information about the hypothetical effector site and to determine which residues are essential for the observed allosteric effects.

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Contract number ERBTS3*CT940319

DISSECTION OF THE MECHANISMS LEADING TO THE SELECTIVE TRIGGERING OF PROTECTIVE AND NON PROTECTIVE MURINE T-CELL RESPONSES FOLLOWING INFECTION WITH *LEISHMANIA*: RELEVANCE FOR THE INDUCTION AND DETECTION OF HUMAN PROTECTIVE IMMUNITY

Period: January 1, 1995 - December 31, 1997

Co-ordinator: INSTITUT PASTEUR, UNITE D'IMMUNOPHYSIOLOGIE CELLULAIRE, Paris, France (G. MILON)

Objectives

To define the early priming conditions that may commit naive/virgin T-cell populations within lymphoid organs draining *Leishmania*-loaded sites to produce a given set of cytokines upon subsequent reactivation in the non lymphoid parasite-loaded tissues such as the dermis. The terms of priming conditions refer to different cellular components: mast cells, mononuclear phagocytes, dendritic leucocytes (mast cells, NK leucocytes, neutrophils, eosinophils, either infected or loaded with parasite extracts as sources of/or exposed to priming cytokines such as IL4, IL12, IFNγ; INFα later named "polarizing cytokines".

To specify the relative contribution of the different cells (a) able to process and present parasite-derived peptides to naive T-cells, i.e. dissect the roles of infected mononuclear phagocytes versus dendritic leucocytes, and (b) able to release a given set of polarizing cytokines. If a peculiar combination of cytokines and antigen-presenting cells is recognized as critical, a logical consequence is to ask whether such priming conditions will be rapidly set in motion *in vivo*, in both the skin site of infection and draining lymph nodes depending upon the genetically dependent ability of mice to control (resistance) or not (susceptibility) the parasitic/pathogenic processes initiated by *Leishmania spp. (L. major, L. brazillensis, L. panamensis).*

To define more relevant correlates of a protective versus non protective immune response within a very well studied polymorphic human population exposed to *Leishmania braziliensis, L. panamensis* while extending the training of scientists in the domain of endemic human leishmaniasis/asymptomatic parasitic processes.

Activities

To pursue *in vitro* studies using naive/virgin and experienced mouse or human T-cells to specify the priming/reactivation conditions that determine whether T cells will produce IL4, IL10, IL13 or interferon γ (IFNγ) upon subsequent rechallenge, namely cytokines which deactivate mononuclear phagocytes rendering them permissive to *Leishmania spp.* growth (IL4, IL10, IL13) or which activate mononuclear phagocytes rendering them no more permissive to *Leishmania spp.* growth (IFNγ).

Contract number ERBTS3*CT940319

To pursue *in vivo* studies in conditions allowing access to both the *Leishmania*-loaded dermis and draining lymph nodes: (ears of mice are optimal sites to inject *Leishmania spp.* and from which to recover all the cells presently thought to be directly or indirectly critical for T cell priming, polarization and for T cell reactivation namely in addition to dendritic leucocytes, mononuclear phagocytic leucocytes and Natural Killer cells, mast cells, neutrophils, eosinophils, keratinocytes).

Expected outcome

A better understanding of the T-cell priming and reactivation conditions set in motion within clinically/epidemiologically defined susceptible and resistant human populations exposed to *Leishmania spp.* The Brazilian and Colombian teams have studied for several years areas where humans are continuously exposed to *Leishmania*. These areas are unique places to study the influence of T-cell responses on outcome of the parasitic process toward either and asymptomatic process or a disease of variable severity.

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Contract number IC18*CT950023

DEVELOPMENT AND IMMUNOLOGICAL EVALUATION OF A VACCINE FOR CANINE VISCERAL LEISHMANIASIS

Period: January 1, 1996 — December 31, 1998

Co-ordinator: ROYAL TROPICAL INSTITUTE, DEPT OF BIOMEDICAL RESEARCH
Amsterdam, The Netherlands (L. OSKAM)

Objectives

- Purif ication of a defined parasite antigen, dp72, for use in laboratory vaccine trials against canine leishmaniasis.
- Development of molecular biological methods, specifically reverse transcriptase — polymerase chain reaction (RT-PCR), to measure cytokine responses, Interleukin (IL) 4, -10, Tumor necrosis factor-α (TNF-α) and Interferon-γ (INF-γ), in vaccinated dogs following infection.
- Examination of Th1 and Th2 responses to vaccine antigens in the experimental model for canine leishmaniasis.
- Identification of a suitable site in Western Turkey where a vaccine field can be carried out.

Activities

* Rever se transcriptase polymerase chain reactions (RP-PCR) for the detection in canine peripheral blood mononuclear cells (PMBC) of lymphokines known to be important in protective and exacerbating responses to Leishmania infections. This will include the measurement of Il-4, IL-10, TNF-α and IFN-γ. PMBC will be developed by standard Ficoll-Hypaque technique using whole dog blood and mRNA isolated by the guanidinium/silica method.
* The antigen dp72 from *L. d. infantum* will be produced for use in vaccination and immunological studies in dogs. Enough protein to vaccinate about 25 dogs (approximately 900 µg) will be purified. The protein dp72 will be purified from a crude membrane fraction of *L.d. infantum* (LV9) by affinity chromatography with a monoclonal antibody (mAb) D13 against the antigen.
* Protection studies in an experimental model for canine visceral leishmaniasis.
* In parallel, dogs with clinical VL will be identified in the field by positive serological diagnosis and tissue biopsies or cultures. Th1 and Th2 T-cell lymphokine expression will be examined by RT-PCR following antigen-specific and mitogen stimulation of PMBL taken from these dogs. This will be compared with the vaccinated dogs as described above.
* Field sites will be identified for vaccines trials. The prevalence of canine leishmaniasis will be examined longitudinally in all the potential field test sites, as described above, during the period preceding the vaccine trial. In addition, a survey of the human population in these areas will be carried out and includes a medical check-up, the DAT and leismanin skin test. This will be used to evaluate the immune status of the dog and human populations at the field trial site.

Results

Attempts to develop an IL-4 RT-PCR based on sequence information from other IL-4s have not been successful. We have postponed IL-4 detection for the assessment of T_H2 response, and continued with IL-10 RT-PCR for monitoring T_H2 response. We have developed a competitive RT-PCR using *in vitro* internal control (IC) mRNA which is added to the sample in order to be able to quantify the amount of RNA present in the sample.

Peripheral blood mononuclear cells (PBMC) from several dogs were tested in this way: one dog was parasitological positive for VL, two dogs were asymptomatic but serologically

positive and one dog was parasitologically and clinically negative at time of sampling but became symptomatic over time.

PBMCs were incubated either in the absence of antigen or in the presence of Leishmania LV9 antigen or ConA. Ten-fold serial dilutions of IC-RNA were added to the RNA isolates. In general, pPBMCs which have been stimulated with either LV9 antigen or Con1 showed a rise in the production of IFN-γ; no significant differences could be observed for IM-10 and TNF-α mRNA levels.

Dogs were vaccinated with Leishmania antigen dp72 plus BCG as an adjuvant. As negative controls they used dogs vaccinated with BCG or saline or nothing at all. PBMCs from these dogs were isolated at five different time points before, during and after vaccination. These samples were stimulated with nothing (negative control), ConA (positive control) or different amounts of antigen.

Preliminary experiments indicate that, in a qualitative RT-PCR using β-actin, IL-10, TNF-α and IFN-γ primers, the only significant difference that could be observed was an increase in IFN-γ levels upon stimulation with ConA. Further quantitation revealed a 100- to 400-fold increase in IFN-γ levels. However, β-actin levels were also increased up to 100-fold after stimulation wit ConA.

We are presently testing more samples and working on the interpretation of the data.

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Contract number ERBIC18CT960079

CHARACTERISATION OF PHOSPHOFRUCTOKINASE AND PYRUVATE KINASE OF *LEISHMANIA*. POTENTIAL TARGETS FOR NEW DRUGS.

Period: November 1, 1996 - August 31, 1998

Co-ordinator: INTERNATIONAL INSTITUTE OF CELLULAR AND MOLECULAR PATHOLOGY (ICP)
Brussels, Belgium (P. MICHELS)

Objectives

- To study the structure and kinetics of phosphofructokinase (PFK) and pyruvate kinase (PYK) of Leishmania, key enzymes in the metabolism of the parasite, and to determine differences with the corresponding mammalian enzymes.
- To design and synthesize selective inhibitors of the Leishmania enzymes, based on their differences with the mammallian enzymes.

Activities

* Cloning and sequence determination of the *Leishmania* PFK and PYK genes.
* Overexpression of the *Leishmania* enzymes in bacteria (*Escherichia coli*) or yeast (*Hansenula polymorpha*).
* Purification of the recombinant enzymes.
* Kinetic analysis of the purified enzymes.
* Structure modelling of the *Leishmania* enzymes, using the X-ray coordinates of the crystal structures of homologous enzymes.
* Structure-function analysis of residues potentially important for inhibitor design by site-directed mutagenesis.
* Crystallization trials of recombinant *Leishmania* PFK and PYK.
* Synthesis of potentially selective inhibitors of *Leishmania* PFK and PYK.

Expected outcome

⇒ The primary structure of *Leishmania* PFK and PYK will become available.

⇒ The enzymes will be overexpressed in a heterologous system and purified.

⇒ Kinetic parameters of the enzymes will be established.

⇒ Preliminary information on the structure of the enzymes will become available.

⇒ Compounds with (some) inhibitory activity on the Leishmania enzymes will be synthesized.

Contract number ERBIC18CT960079

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Contract number IC18CT960084

ANTILEISHMANIAL AND ANTITRYPANOSOMAL ACTIVITIES OF ALKYL-LYSOPHOSPHOLIPIDS

Period: 1 October 1996 - 30 September 1999

Co-ordinator: LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE, London, United Kingdom (S.L. CROFT)

Objectives

- To determine the mechanisms of action of alkyllysophospholipids (ALPs) against *Leishmania* and *Trypanosoma cruzi*, including effects on the host immune response
- To identify novel biochemical and molecular targets in *Leishmania* and *Trypanosoma*
- To establish ALP resistant clones and identify mechanisms of resistance
- To define interspecies and interstrain variation in sensitivity to ALPs
- To define a structure-activity relationship of the antileishmanial and antitrypanosomal activities of ALPs as they represent a new selective model for further antiprotozoal drug development
- To define rational drug combinations to be used in treatment.

Activities

* The activities of ALPs, alone and in combination with other drugs, will be determined against both exacellular and intracellular forms of different strains/species of *Leishmania* and *Trypanosoma* by microscopical and biochemical techniques.
* The effects of ALPs on membrane pathways, in particular sterol, lipid and glycosylphosphatidylinositol (GPI) anchor biosynthesis, will be studied.
* Effects on parasite differentiation and signal transduction will be examined in particular in relation to roles of protein kinases, phospholipases, calcium levels and adenylate cyclase.
* The uptake and distribution of ALPs by parasites and host cells will be measured by isotopic and chromatographic methods.
* Immunomodulating properties of ALPs will be studied in relation to killing of intracellular stages of *Leishmania* and *Trypanosoma cruzi* in macrophages.
* Resistant clones of *Leishmania* and *Trypanosoma cruzi* will be established through the stepwise exposure of extracellular parasites to increasing concentrations of ALPs.

Expected Outcome

⇒ An understanding of the mechanisms of activity of a novel group of antiprotozoal drugs and the identification of novel drug targets
⇒ Data on drug activities and drug combinations useful for clinical studies on leishmaniasis and trypanosomiasis
⇒ PhD students will be trained in laboratories in Europe and South America and international links cemented.
⇒ Results will be published in international journals, presented at international meetings and be the central focus of an EC meeting to which representatives of pharmaceutical companies will be invited.

Contract number IC18CT960084

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Contract number ERBIC18CT960123

CLINICAL VARIABILITY OF AMERICAN TEGUMENTARY LEISHMANIASIS IN PERU AND BOLIVIA: RELATIONSHIP WITH POLYMORPHISM OF THE PARASITE WITHIN THE *LEISHMANIA BRAZILIENSIS* COMPLEX OF SPECIES (SYN. SUBGENUS *VIANNIA*)

Period: October 1, 1996 - September 30, 1998

Co-ordinator: PRINCE LEOPOLD INSTITUTE OF TROPICAL MEDICINE, PARASITOLOGY DEPARTMENT, Antwerpen, Belgium
(JEAN CLAUDE DUJARDIN, DOMINIQUE LE RAY)

Objectives

To identify parasite characters linked with variability in clinical response to infection with parasites of the *Leishmania braziliensis* complex in Peru and Bolivia:

- To collect and analyse comparable clinical and epidemiological data from three endemic areas in the Amazon basin of Peru and Bolivia in order to discriminate factors underlying clinical variability in infection and disease.
- To strengthen the argument that there is apparently a consistent association between parasite genetic variation, virulence and clinical variability.
- To initiate experimental studies on biological parameters that might be involved in *Leishmania* virulence, by using isolates with well defined genotypes obtained from representative clinical categories.

Activities

* To collect comparable epidemiological and ecological data in the two previous STD3 (LEISHBOLPE 3) study areas (Pilcopata, Amazonian foothills, Peru; Isiboro Secure, Amazonian lowlands, Bolivia) in reference to the methodology already developed for Huanuco Valley (Peru).
* To quantify clinical variability of both cutaneous and mucosal stages of infection and disease by data collection with the same standardised gradings in the 3 study areas (Pilcopata, Isiboro Secure, Huanuco).
* To undertake epidemiometric statistical analysis of field data for discriminating factors underlying clinical variability in infection and disease.
* To collect both biopsy samples and parasite isolates from lesions of patients documented above, for activities described below.
* To pursue genomic and genetic characterization of parasites according to clinical grading.
* To qualify the predictive value of candidate genetic markers associated with specific clinical and phenotypic patterns by:
  - Analysis of the genetic structure of parasite populations under study.
  - Characterization of identified marker chromosomes and corresponding nucleotide sequences.

  Validating the operational value of corresponding probes and primers on the collection of biopsy samples (cryobanked) if time and tools permit.
* Complete molecular characterization of isolates collected by the previous project LEISHBOLPE 3 until isolates considered herein (LEISHBOLPE 4) become available.
* Developing biological *in vitro* and *in vivo* characterization of a limited set of parasites, preferably cloned, representative of the genetic and clinical categories identified above.
* Assessing Leishmania survival inside the macrophage as a measurement of parasite virulence.
* To process the data for global analysis of correlations between genetic and clinical variability.

Contract number ERBIC18CT960123

Expected outcome

⇒ Comparable epidemiological, ecological and clinical data in the three study sites of Peru and Bolivia (Andean valleys, Amazonian foothills, Amazonian lowlands).
⇒ Collection of parasites from isolates and in biopsies, characterized genomically and genetically according to clinical grading.
⇒ *In vivo* and *in vitro* models for biological characterization of parasite virulence.
⇒ Discrimination of factors underlying clinical variability in infection and disease.
⇒ Documenting whether there is a correlation between parasite genetic variability and clinical variability. If there is a correlation, validation of (i) corresponding risk factors for patient's care, and of (ii) corresponding probes and primers for parasite identification in field biopsies.
⇒ Genetic structure of parasite populations in the three study sites.

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Contract number IC18*CT970213

HOST-PARASITE RELATIONSHIP IN CANINE VISCERAL LEISHMANIASIS (*L. INFANTUM/L. CHAGASI*): DEVELOPMENT AND VALIDATION OF THE DOG MODEL

Period: January 1, 1998 - December 31, 2000

Co-ordinator: INSTITUTO DE SALUD CARLOS III, Centro Nacional de Microbiología, Majadahonda, Spain (J. ALVAR)

Objectives

- To define the kinetics and characteristics of infection, immune response and clinical evolution of *Leishmania infantum* infection in the dog, the main reservoir of visceral leishmaniasis.
- To describe the natural history of canine leishmaniasis in inbred dogs, with emphasis on the prepatent period
- To develop reagents that may have prognostic value.
- To develop a model useful for future immunoprophylactic and therapeutic studies and to establish a reproducible infection protocol that results in consistent parasitological, immunological and clinical patterns.
- To compare and validate the experimental model with natural infection in endemic areas (New and Old World). Validate the experimental model by comparing clinical, immunological and parasitological data of asymptomatic dogs (defined by parasite burden, and biochemical analysis) from endemic areas in Colombia and Spain.

Activities

The natural history of canine leishmaniasis in inbred dogs will be investigated with emphasis on the prepatent period by:

* Clinical analysis including analytical biochemistry and blood cell count.
* Measurement of systemic and cutaneous cell-mediated responses by studying immunocytochemical characterization of the first and second signals of lymphocyte activation. Cytokines will be measured and the specific response to defined antigens will be studied and T-cell subsets established.
* Investigation of the immunostimulatory and effector roles of dendritic cells in a canine model of visceral leishmaniasis.
* Measurement of antibody isotype responses in particular IgM, IgG and IgE
* Parasite burden and parasite distribution will be established by direct microscopy, by culture in NNN and by PCR.
* Infectivity for sandflies (epidemiological risk) will be assessed at different time points by Xenodiagnosis with *Phlebotomus perniciosus* or *Lutzomyia longipalpis* and asymptomatic and symptomatic dogs from endemic areas (Spain and Colombia).

Reagents that may have prognostic value including PCR primers for IL-4, IL-10, IL-12, and TGF-β will be developed together with a quantitative PCR of genomic DNA for determining parasite burden.

Additional work will attempt to develop a model useful for future immunoprophylactic and therapeutic studies with the aim of establishing a reproducible infection protocol that results in consistent parasitological, immunological and clinical patterns. The experimental

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model will be validated with natural infection in endemic areas (New and Old World) by comparing clinical, immunological and parasitological data of asymptomatic dogs (defined by parasite burden, and biochemical analysis) from endemic areas in Colombia and Spain.

Expected outcome

⇒ A better understanding of the infection on the basis of correlating the immunobiology, clinical and epidemiological features.
⇒ Practical recommendations will result from this project in terms of risk for humans and control measures through a better understanding of the natural history of canine leishmaniasis.
⇒ Moreover, the response to defined antigens will establish the immunological basis for further projects related to vaccine development or for drug/immunological synergy.
⇒ Finally, several reagents are expected to be obtained for both diagnosis and cytokine detection, that will be of value for the scientific community.

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Contract number IC18*CT970220

NEW TRYPANOCIDAL COMPOUNDS BASED ON INHIBITORS OF GLYCOLYSIS AND THE SPECIFIC IMPORT OF THESE INHIBITORS INTO THE PARASITE

Period: October 1, 1997 - September 30, 2000

Co-ordinator: UNIVERSITE PAUL SABATIER, Groupe de Chimie Organique Biologique, Toulouse, France (J. PERIE)

Objectives

- to develop novel compounds active against human trypanosomiases and leishmaniasis;
- to take advantage of an essential metabolism in these trypanosomatidae that glycolysis represents;
- to design irreversible and quasi-irreversible inhibitors of glycolytic enzymes based on differences between parasites and mammalian enzymes and to import them into the cell either via passive diffusion or via the glucose THT1 transporter.

Activities

The first set of irreversible GAPDH inhibitors will be extended to structures directed towards Arg 231 and the compounds will be transformed into the corresponding prodrugs. Cocrystallisation experiments of these compounds with the enzyme GAPDH from *Trypanosoma brucei* will be made for the design of improved structures after corresponding modelling.

A parallel work will be made on the *T.brucei* aldolase enzyme, starting from active structures already identified. In both cases the residue responsible for the formation of the covalent bond will be identified using mutants for the most likely locations.

All the compounds will be assayed for their transport by the glucose transporter THT1 and also on Trypanosome cytosolic esterases which are expected to transform prodrugs into drugs within the cell. Compounds of natural origin will be also studied within the program.

Expected outcome

The study will develop compounds capable of specifically blocking glycolysis and therefore limiting the survival of trypanosomes. The work will lead to an improved knowledge of the glycolytic enzymes and transport systems in trypanosomes and leishmania.

Contract number IC18*CT970220

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A NETWORK APPROACH TO RESEARCH ON LEISHMANIASIS IN CENTRAL AMERICA WITH EMPHASIS ON DRUG SENSITIVITY IN THE FIELD

Period: October 1, 1996 - January 31, 1998

Co-ordinator: KEELE UNIVERSITY, DEPT. OF BIOLOGICAL SCIENCES, Staffordshire, United Kingdom (R. WARD / R. MAINGON)

Objectives

- To obtain information on the nature and mechanisms of drug sensitivity in the field using a controlled prospective population-based study.
- To improve diagnosis and parasite/vector identification in Central America using traditional and new molecular methods.
- To improve human resources with expertise on leishmaniasis using a network approach.

Activities

The project focuses upon an examination of drug sensitivity to glucantime by the *Leishmania* species circulating in Guatemala and other American countries. Genetic analysis of glycoprotein genes in Granada, Spain is complemented by work at Keele, UK using broader molecular approaches such as differential display. In Nicaragua and Panama PCR methods will be applied to examine for post treatment parasite persistence. Studies in Honduras and El Salvador are focused upon isolation of new strains of *L. chagasi* from typical and atypical clinical cases along with preliminary studies on transmission dynamics in the San Juan Bautista and Choluteca areas.

Results so far

During the first year of the project a modified work plan was drawn up as a result of a preliminary meeting in Guatemala in January 1997. Specific gene expression patterns in *Leishmania* are being studied. Using the mRNA differential display technique putative species specific transcripts of *L. mexicana* and *braziliensis* have been obtained. Furthermore transcripts specific to a glucantime resistant cloned *L. tropica* have been produced. Several members of th group have participated in training exchanges with partner laboratories and have participated at the First World Congress on leishmaniasis held in Istanbul during May 1997.

A single batch of 5500 glucantime ampoules were obtained from France and are being used in Guatemala in the patient cohort study. *Leishmania* isolates from patients in El Salvador have been characterised in Honduras and the strains passed to the UK laboratory and Nicaragua. In Panama studies are revealing the wide diversity of treatment regimes which make evaluation of drug sensitivity a difficult parameter to monitor. Blood samples and aspirates of lesions from the Penonome endemic area have been carried out and results are expected in the near future.

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A member of the Panamanian group has received training in PCR techniques in the Nicaraguan laboratory. The Nicaraguan group have also recorded the occurrence of atypical visceral leishmaniasis similar to those cases recorded from Honduras and Costa Rica. The predominant vector in this area was *Lutzomyia longipalpis*.

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Contract number IC18*CT970225

PRODUCTION AND CHARACTERISATION OF SYNTHETIC INHIBITORS OF PARASITE OF PROTEASES AS DRUG CANDIDATES FOR THE PREDOMINANT PROTOZOAL DISEASES OF SOUTH AMERICA AND OTHER DEVELOPING REGIONS

Period: October 1, 1997 - September 30, 2001

Co-ordinator: CARLSBERG LABORATORY, DEPT. OF CHEMISTRY,
Valby, Denmark (M. MELDAL)

Objectives

To develop specific inhibitors of parasitic cysteine proteases as drug candidates for the treatment of the predominant protozoal diseases (in particular *Leishmaniasis* and Chagas disease) of South America and other developing countries. This goal will be attained through the implementation of molecular biology, enzymology and state of the art combinatorial chemical library techniques.

Activities

Solid phase chemical synthetic methodology will be developed simultaneously with molecular and recombinant technology which will generate large quantities of cysteine proteases for screening. An iterative process of screening and optimization will lead to the target drug candidates. The measurable objectives are outlined as follows in quasi-chronological order.

* The development of resins suitable for both synthesis of non-peptide inhibitor libraries as well as solid fluorescent-quenched enzymatic assays.
* The isolation of recombinant and native *Leishmania* cysteine proteases (CPs) in large amounts.
* The determination of substrate specificity of recombinant cruzipain and important *Leishmania* CPs using substrate libraries and MS detection.
* The development of several methods (nanoprobe MAS-NMR of single beads, MS-MS, and ladder synthesis/MS) for the fast and efficient analysis of resin-bound peptide substrates and non-peptide inhibitors.
* The development of solid phase synthetic methodologies for the rapid generation of potent and selective CP inhibitors.
* The construction of a novel syntheziser for manual organic library synthesis with the capacity to provide inert reaction conditions, for temperature control and for refluxing conditions.
* The screening of substrate and inhibitor libraries with *T. cruzi* and *Leishmania* CPs and the detection of potent inhibitors.
* The synthesis of inhibitors in larger quantities and an analysis of their activity towards pure CPs. The selectivity of the inhibitors will be assessed by comparison with mammalian cathepsins B and L.
* The testing of inhibitors for antiparasite activity *in vitro* and *in vivo*.
* The application of gene knockout and over-expression of CPs in *L. mexicana* and subsequent transfer of that methodology to other *Leishmania* species in order to identify and characterize key CPs and to produce recombinant enzymes for use in drug development.

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Another important aspect of this project is the transfer of knowledge and techniques required for synthesis and enzymatic assays of chemical libraries and methods of reverse gene technology to partners in developing countries. The transfer will partly be established by training visits in the EU-based laboratories. In addition, a multidisciplinary research network in LA-EEC will be established to foster the development of the field of drug leads and design using an interdisciplinary approach involving state-of-the-art molecular and biochemical techniques and combinatorial chemistry.

Expected outcome

Since the chemotherapeutic treatment of parasitic diseases is not satisfactory because of drug toxicity and the development of drug resistant parasites, the development of alternative drugs is of grave import. By the end of this project, we will have developed a new class of antiparasitic drugs. Simultaneously, we will have deepened the understanding of the mechanism and specificity of CPs and the mode of action of various parasites. Moreover, we will have established a multidisciplinary, international research network to foster innovations in the drug discovery approach.

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Contract number IC18*CT970252

MOLECULAR GENETICS, CELLULAR IMMUNOLOGY AND PEPTIDE-CHEMISTRY FOR THE DEVELOPMENT OF CONTROL TOOLS AGAINST LEISHMANIOSIS

Period: October 1, 1997 - September 30, 2000

Co-ordinator: INSTITUT PASTEUR, UNITE DE GENETIQUE MYCOBACTERIENNE
Paris, France (B. GICQUEL)

Objectives

There are three main objectives:

- The study of the biological function and role in the *Leishmania* infectivity of two molecules encoded by developmentally regulated *Leishmania* genes, namely a putative nuclear growth related factor (LNP18) and an immunodominant membrane protein.
- The dissection of the cellular immune responses conferring resistance to *Leishmania* spp. driven pathogenic processes.
- Development of new reliable diagnostic products for early detection of leishmaniosis.

This project aims at identifying parasite factors essential for *Leishmania* infectivity and at dissecting cellular immune responses conferring resistance to *Leishmania* spp.-driven pathogenic processes. Such advances should pave the way for the development of new strategies and the rational design of future immunoprophylactic and immunotherapeutic measures to control leishmaniosis. Leishmaniosis constitutes not only a major public health problem of socio-economic importance but is also a veterinary problem in various parts of the world and especially in Mediterranean countries. Thus, there is a need for the development of new effective tools for their control. Recent advances in the field of molecular genetics, cellular immunological tools that the European partners have recently developed for characterizing and studying parasite and host factors promoting or circumventing the development of the disease. The rational approach that will be pursued is based on the use of all these tools focusing on two *Leishmania* molecules, a putative nuclear growth related factor (LNP18) and a B-cell immunodominant antigen (P32) that induces also T cell responses encoded by two novel genes recently identified by partners n°2 and n°4 respectively, for the elucidation of (a) their functions in pathogenesis (b) the nature of molecular and cellular effectors/regulators on which they act as sites of *Leishmania* delivery or spread (c) their impact on the selective activation and differentiation of the CD4+ and CD8+ T-cell subsets to drive a protective Th1-cell response and (d) their potential use as reagents for diagnosis. The immunogenic potential of the different Leishmania molecule formulations will be studied in the murine model and the recently established by partners n°3 and n°5, canine models. Such understanding will allow the design of rational approaches to combat leishmaniosis.

Activities

In particular, we propose to:

* Develop *Leishmania* mutants by sequential targeted gene replacement or site directed mutagenesis.
* Determine the phenotype conferred by LNP18 and P32 mutations.
* Evaluate the role of LNP18 and P32 in parasite infectivity.

The study will:

* Investigate the host-protective capability of defined *Leishmania* antigens. New protective antigens and two B immunodominant molecules LNP18 and P32 will be identified,

cloned, sequenced and characterized, using recombinant molecules as well as synthetic peptides modelled from their respective amino-acid sequences.

* Determine ways and parameters of directing the immune system towards a Th1 type response, by examining both CD8+ and CD4+ lymphocyte responses, using vectors such as live recombinant BCG expressing *Leishmania* antigens e.g. LNP18 and P32, and synthetic microspheres carrying these antigens.
* Conduct protection studies in canine experimental models.

More specifically, we will evaluate the diagnostic potential of:

* recombinant LNP18 and P32
* synthetic peptides modelled from P32 and LNP18 amino acid sequences, as targets for ELISA. Data on P32 and on a LNP18 20-peptide make these molecules promising reagents for serodiagnosis.

Expected outcome

Following completion of the project, the results obtained will be sufficiently robust for the industrial environment and will provide new strategies and tools to help the leishmaniosis control such as: *Leishmania* mutants and live recombinant BCG expressing *Leishmania* antigens, as possible vaccine candidates, host-protective parasitic synthetic peptides and recombinant molecules as well as particulate antigens, and reliable diagnostic tools.

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Contract number IC18*CT970256

INVESTIGATIONS ON POLYMORPHIC GENOMIC MARKERS IN RELATION TO APPLIED FIELD RESEARCH ON THE BIOLOGY OF LEISHMANIA PARASITES IN VARIOUS ECO-EPIDEMIOLOGICAL SETTINGS IN THE MEDITERRANEAN BASIN

Period: November 1, 1997 - October 31, 2000

Co-ordinator: INSTITUT PASTEUR DE TUNIS, LABORATOIRE D'EPIDEMIOLOGIE ET D'ECOLOGIE PARASITAIRE
Tunis Belvédère, Tunisie (I. GUIZANI)

Objectives

To provide genomic tools to measure the extent of DNA polymorphisms among natural *L. infantum* parasite populations and to approach underlying molecular mechanisms to parasite populations diversity; intra-chromosomal recombination; chromosomal assortment, gene rearrangement and chromosomal size variability. These DNA tools will be applied in at least three eco-epidemiological settings in the Mediterranean area: France, Lebanon and Tunisia. In particular, molecular tools will be used to complement field studies in Lebanon which aim at better defining the epidemiology of leishmaniases in this country.

Activities

* DNA markers for polymorphisms will be identified using different approaches: chromosomal size variability, variations in copy numbers, RFLP and sequence analysis. Newly identified markers will be submitted to extensive molecular analysis which will include mapping and sequences.
* Repeated coding sequences (gp63, PSA2, rDNA, miniexons) will be targeted to look for RFLPs and to study variations in copy numbers in relation to chromosomal size variability.
* PCR tests will be developed based on the gp63 gene family, minisatellites and microsatellites. A thorough screening for microsatellites on chromosome V will be made using a sequencing approach. Evaluation of these PCR tests will be performed on promastigote populations that will be the subject of this study and on the biological material that will be collected from patients and dogs in Tunisia and Lebanon.
* The study of the mechanisms underlying the genetic diversity in natural populations of parasites will be done on parasite populations collected in close sympatric. Intrachromosomic recombination will be studied on chromosome V. The chromosomal assortment will be studied by using unique markers to defined chromosomes (RFLP and/or PCR tools).
* Correlations between chromosomal size variability and variations in copy number of target genes will be investigated. These results will also be correlated to clinical outcome of the parasite infection: cutaneous leishmaniasis (CL) vs. visceral leishmaniasis (VL). To avoid biases which could be encountered while working on closely related parasite populations, this study will be conducted on groups of parasites from sympatric VL and CL cases, isolated in different geographical areas.

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Expected outcome

A better understanding of the epidemiology of leishmaniasEs in Lebanon is expected at the end of this study. This will provide a better identification of the causal agents. Earlier preliminary studies have pointed to the possible occurrence of the *L. donovani* complex parasites in this country. It will be particularly interesting to understand why CL is predominantly recorded in this area. PCR tools for studying parasite polymorphisms constitute an innovative approach which will provide new insights in the field situation and on the molecular epidemiology of leishmaniases caused by parasites of the *L. donovani* complex. The study will provide well characterized DNA tools. Such tools will allow a better understanding of the parasite genetics and thus will help in defining parasite determinants involved in the biology of the Leishmania parasites.

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Presentation of EC supported joint research projects (1991-1996) continued
STD3
INCO-DC: 1st and 2nd Call

Areas of interest:

5. Filariases

Contract number: ERBTS3*CT910031

DYNAMICS OF LYMPHATIC FILARIAL INFECTION AT THE CELLULAR AND MOLECULAR LEVEL

Period: April 1, 1992 - March 31, 1995

Co-ordinator: IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY & MEDICINE, DEPT. OF BIOLOGY, London, United Kingdom (R. MAIZELS)

Objectives

- To study the dynamics of the host-filarial interaction over time in individuals and populations using established indicators of infection, T cell activation and antibody production.
- To study the role of T cells in immunity and pathogenesis in human filariasis:
  (a) as a function of immune status and clinical category of individual patients
  (b) in terms of lymphocyte regulation and activation by cytokines and antigen presenting cells.
- Training of scientists from, and strengthening of research capability at, the University of Indonesia and consolidation of the long-standing collaborations between institutes in Indonesia, the Netherlands and the UK.

Summaries of achievements

In the period covered by this project, it has been possible to carry out a unique longitudinal study of subjects resident in an area endemic for lymphatic filariasis in Central Sulawesi, in Indonesia. The immunological changes as measured by antibody isotypes and cellular reactivities have been documented in individuals who were categorized as asymptomatic amicrofilaraemics and asymptomatic microfilaraemics. It has become clear that both humoral and cellular immune responses are strongly modulated by filarial parasites and that the changes in parasite density can affect the immune parameters such as proliferation and cytokine production. The studies point toward a role for IFN-γ and IL-5 in immunity towards filarial parasites.

Activities

* Contact of Indonesian team with the authorities of The Centre for Disease Control in Sulawesi to initiate the field study. Preliminary visits to the study area (two villages in the area of Langa Leso in the Palu Valley in Central Sulawesi) to organize health education programme which would inform the villagers not only about the project and its aims but also about filariasis in general. Recruitment of the members for the local team in charge of the project. One member of staff from Jakarta was housed for two years in the area for continuous monitoring of the study.
* Screening of the inhabitants of two villages by questionnaires, by night blood filtration and by clinical assessment. A detailed map of the village was constructed and each member of the house was registered alongside its parasitological and clinical status (if the inhabitants had taken part in the survey).

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* Individuals giving informed consent and selected for participation in the project were asked to donate blood at 3-4 month intervals. Individuals younger than 16 were asked to give blood only at the start and one year later at the end of the study period. Any person who developed symptoms related to filarial infection was treated with DEC and the excluded from the study.

* The presence of microfilariae was determined in 1 ml night blood at time points over one year. Also, 20mls of blood was drawn for immunological studies. The blood was transported to Jakarta for fractionation into plasma and mononuclear cells.

* Isolated cells were cultured for antigen and mitogen stimulated cytokine production and the rest of the cells were cryopreserved for use at a later time. The plasma was used to perform tests in Jakarta (anti-L3 surface antibody detection, immunoblots) and in Edinburgh/Imperial College (antibody isotype ELISA and immunoblots). Cells and culture supernatants were used for detecting proliferation and cytokine production as well as generation of T cell lines.

* Parasite antigens were cloned and used for antibody isotype reactivity by ELISA. The analysis of IgG4 reactivity in particular to parasite antigens was also determined by immunoblots and IFAT.

* Culture supernatants were tested for the presence of IL-4, IL-5, IL-10, IL-13 and IFN-γ. Cryopreserved cells were used to generate T-cells for TCR-Vβ gene analysis at the molecular level.

Results

Immunological Profiles of Individual Infected with Filarial Parasites

It has become clear that individuals infected with *Brugia* malayi show elevated IgG4 against filarial antigens in the face of relatively low specific IgE. If worms are cleared by DEC treatment, IgG4 levels decline within a period of 12 months whereas anti-filarial IgE responses remain largely unchanged.

Thus, the presence of filarial parasites is a major stimulus for the extremely high levels of IgG4. At the cellular level, infected individuals show a defect in T-cell proliferative responses to filarial antigens. In terms of cytokines, IFNγ production in responses to parasite antigen appears to be low in Mf carriers whereas cells respond to antigen by releasing IL-4, IL-5, IL-10 as well as IL-13. In one study it was possible to look at proliferation as well as IFNγ and IL-4 production before and after chemotherapy. After treatment both T-cell proliferation and IFNγ production increased, whereas IL-4 remained unchanged. These studies indicate that exposure to parasites leads to T-cell proliferative unresponsiveness as well as suppressed IFNγ production.

By comparing Mf-positive individuals with Mf-negative ones, it is possible to determine which responses are altered by high parasite densities. We have confirmed that IFN-γ is suppressed in Mf-positive persons, but we also noted that IL-5 production can be significantly lower in infected individuals whereas IL-4 production was the same when all age groups were considered together.

These results have lead us to the conclusion that not only TH1 but also selective TH2 type responses can be suppressed by the presence of filarial parasites. This has important implications for the design of prophylactic measures as appropriate cytokines must be stimulated for successful parasite elimination.

Changes in Anti-Filarial Immune Responses as a Function of Age

As the length of exposure to parasites or age can be an important factor in determining the level of immunity to a parasitic infection, we have examined the changes in the specific antibody levels as well as cytokines in an age stratified manner. During the initial survey we collected 400 serum samples and have consequently performed ELISAs for the determination of anti-filarial antibody isotypes. The study has shown that anti-filarial IgG4 increases with age and reaches a plateau in early adulthood. The pattern of other isotypes is less clearly associated with age. The increases in IgG4 may indicate the acquisition of increasing parasites by an individual. The results could also be interpreted as a measure of increasing exposure to incoming L3, but the decline of IgG4 after chemotherapy means that the presence of worms contributes to the elevated IgG4. In a subset of the study subjects it was possible to analyze IL-4, IL-5 and IFNγ as a function of age. When considered together, IL-5 was the only cytokine that seemed to decrease as a function of age. However, when segregated into Mf+ and Mf-, there was a profound effect of age on the cytokine release profiles. IFN-γ increases with age in asymptomatic amicrofilaraemics, whereas IL-4 increases with age in Mf positive individuals. These results might indicate that IFN-γ could play a role in immunity to incoming larvae when a person remains free of parasites, whereas IL-4 might be important in immunity to incoming larvae when a person is infected with adult worms.

Fluctuation in parasite densities and immunological parameters

It has been possible for the first time in lymphatic filariasis to monitor longitudinally parasitological changes and the accompanying immunological responses. Study participants were screened 3 times throughout one year (at 4 monthly intervals) for the presence of Mf in 1 ml night blood. The levels of Mf fluctuated considerably, starting at relatively high levels in April, to significantly lower levels in July, with a subsequent increase in November. The reason for the observed fluctuation is not clear. Such fluctuations have been reported before and are thought to accompany climatic changes. As the adult worms live more than 5 years and the time before an L3 becomes and adult worm is more than 3 months, the fluctuation in Mf density is most likely a result of change in the reproductive activity of the female worms. It is possible that when the number of biting mosquitoes is low, it would be more advantageous for the parasite not to produce many microfilariae. July marks the end of the dry season in the area when numbers of mosquitoes is low and indeed this might be the reason for low levels of Mf seen in the study population. Although at the present we are unable to clarify the Mf fluctuation, it is evident from our study that immunological parameters can also alter. The antibody levels remained fairly constant throughout the study period. However, T-cell responses fluctuated considerably. T-cell proliferation was low in April, became significantly higher in July and decreased again in November. This indicates that an increase in reproductive activity of the female worms might be associated with a more profound suppression of the T-cell proliferactive responses to filarial antigens.

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IgG4 antibodies in Brugian filariasis

A comprehensive qualitative and quantitative analysis of IgG4 antibodies in filarial infection has been possible through this project. Thus, it was shown that parasite-specific IgG4 is preferentially directed against low molecular-weight antigens relative to other isotypes, and 5 individual target proteins were identified. Some of these correspond directly to known recombinant antigens, and others were new.

Over the course of the longitudinal study, we have measured levels of each isotype of anti-filarial antibody as well as microfilaraemic status. We now have data indicating that high IgG4 levels (measured against total BmA) in amicrofilaraemic patients are not necessarily prognostic of a future conversion to Mf-positivity, and conversely that some low-IgG4 Mf-negative cases do convert to Mf-positive within a few months. This finding indicates that serodiagnosis cannot rely on total IgG4 levels, but will require more restricted antigenic specificity afforded by recombinant antigens.

We have also completed a study on the isotype of antibodies binding directly to the surface of the infective larva (L3) stage, presumed to be the target of protective immune responses. Most interestingly, little of this antibody is of the IgG4 isotype, although that subclass predominates among antibodies reactive to whole worm (larval and adult) antigens. This strengthens the case that the L3 surface contains exclusive specificities, not present in later stages, which may be good candidates for vaccine development.

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Contract number ERBTS3*CT920141

STUDIES ON THE DEVELOPMENT OF PROTECTIVE IMMUNITY IN LYMPHATIC FILARIASIS CAUSED BY *BRUGIA MALAYI* IN AN AGE-GRADED POPULATION

Period: January 1, 1995 - May 31, 1996

Co-ordinator: LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE
London, United Kingdom (D. DENHAM)

Objectives

To analyze immune responses towards filarial antigens in a well characterized population resident in areas endemic for lymphatic filariasis. Parasitological, clinical and immunological markers of infection, when correlated with age, can yield important information about the dynamics of the host parasite interaction. Comparison of infected versus uninfected as well as children versus adults should identify immune pathways that operate to decrease susceptibility to infection in either the uninfected individuals or in subjects who resist incoming L3, despite harboring adult worms. Both antibody isotype profiles and cytokine levels released in response to parasite antigens (*in vivo* as well as *in vitro*) can indicate the extent to which TH1 and TH2 cell subsets are reactive in lymphatic filariasis. The exact contribution of these two subsets to immunity or pathogenesis of filarial infections has yet to be delineated. As the fine specificity of immune recognition is determined by MHC, the HLA typing of the study population will be necessary for our complete understanding of immune reactivity in filarial infected subjects. As far as our understanding of immunity to filarial parasites is concerned, our objectives can be summarized as follows:

- Extensive epidemiological surveys to gather precise demographic information and to assess parasitologic and clinical status of the study subjects.
- HLA typing of the study population to correlate infection status and disease with HLA.
- Antibody isotype analysis in the study population with particular emphasis on IgG4 and IgE, isotypes that are elaborated in helminth infections and seem to play an important role in resistance or susceptibility to schistosome infections.
- Cytokine profiles in serum of the study population in order to correlate their levels with infection status.
- Cytokine production by PBMC in response to parasite antigen to understand the TH1 versus TH2 balance in clinical groups as a function of age.

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Activities (so far)

The coordination of the project is now from the University of Leiden and a new developing country partner Hasanuddin University in Indonesia has been included. Dr. Medeiros started her training to become a pathologist and therefore some of the Portuguese tasks were performed in Leiden on their behalf.

* A second field trip to Sulawesi was undertaken in July 1995. Two of the members of staff from Leiden University and 3 staff members from Hasanuddin University were in charge of the field activities. A continuation of the screening of villages involved completion of questionnaires, night blood filtration (10ml) for detection of microfilariae and complete clinical examination. The study has examined in total 248 individuals with an age range of 7 to 80 (see results for study population description).

* HLA analysis of the study population was carried out by oligotyping.

* Measurements of anti-filarial IgG4 and IgE.

* Total IgG4 and IgE levels were determined and related to the specific antibody levels in order to understand how these two isotypes are regulated as a function of age at the polyclonal as well as antigen specific level.

* Anti-filarial IgG1, 2 and 3 levels were measured and expressed as a function of clinical status and age.

* Serum levels of IL-4, IL-6, IL-8, IL-10 and IFNγ were measured. In addition, the levels of LPS-binding protein, ICAM, E-selectin and TNF receptors I and II were determined to assess the extent of granulocyte activation as well as stimulation of inflammatory cytokines in filarial infections.

* For the assessment of age graded T cell responses, it proved to be more feasible to carry out the study in Central Sulawesi where an ongoing CEC-supported project (CT1-CT1-0928 and TS3*CT920031) enabled the use of facilities that are needed for cell work. In the meantime, the laboratory at Hasanuddin has established an operational laboratory with flow hood and centrifuges that would enable future cell work. IL-4, IL-5 and IFNγ production by PBMC was measured in response to filarial antigens and analyzed as a function of age.

* A proportion of the infected individuals participated in DEC chemotherapy. The treatment was administered for 10 days and repeated 3 times at 2 week intervals. The antibody levels were followed and a study is now set up, for the first time in filariasis, to address the issue of reinfection.

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Achievements so far

The study area is the north of Mamuju in South Sulawesi. The study population consisted of 248 individuals with a mean age of 25.7 (range 7-80). Mean length of residence was 14.9 years (range 1-80). 26% were mf+, 75% IgG4E and 64% IgE+. GMmf count was 241. A subset of these individuals have been typed for HLA class I and II. So far the data have been analyzed to search for correlates between HLA and elephantiasis. No significant and reproducible correlations could be found. The use of anti-filarial IgG4 for determining the degree of endemicity of *Brugian* filariasis indicates that this test can indeed be a useful and more sensitive substitute for the night blood sampling. The levels of anti-filarial IgG4 have been analyzed as a function of gender to show that males and females show consistent differences in infection pattern.

Filarial infections increase as a function of age in both males and females. Males appear always to be infected to a greater extent than females (irrespective of age or clinical status). The level of anti-filarial IgE in the study population indicates that microfilaraemics show depressed levels of IgE when compared with mf negative subjects. The data are now being analyzed as a function of age and gender. Although the analysis is not yet complete, it appears that anti-filarial IgE is high in young subjects exposed to filarial infections and decreases with increasing age. However, individuals aged 50 or older show increases in levels of IgE directed to filarial parasite antigens. Although all the ELISAs for anti-filarial IgG1, 2 and 3 have been performed, the data have yet to be analyzed.

The levels of inflammatory cytokines as well as acute phase reaction proteins have been analyzed in a subset of the study subjects. It was found that there was a significantly higher levels of IL-6 in individuals with elephantiasis than in microfilaremics or amicrofilaremic individuals. No differences were seen in any of the other parameters mentioned above. The response of PBMC to filarial antigen in terms of IL-4, IL-5 and IFNγ production was also analyzed in order to assess how age and clinical status influences the balance between these cytokines. It is clear that the presence of mf suppresses both IL-5 and IFN-gamma leaving IL-4 unabated. Age was shown to have a significant effect on profiles of cytokines released by different clinical categories. However, we were unable to observe any correlates with concomitant immunity.

Finally, the IgG4 ELISA is now operational in Ujung Pandang and the research team will be able to carry out their own surveys for control programmes.

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An additional field trip is planned for sampling of blood from young children (<10 years).

The blood will be collected onto filter paper. The levels of IgG4 in particular will be assessed. If possible, the levels of IgE will also be determined. The individuals who have participated in DEC chemotherapy will be followed for re-infection as well as antibody reactivity. Once the re-infection data is available, the immunological reactivities measured will be analyzed within a framework of immunity to filariasis taking into account age, sex and clinical status.

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Contract number IC18CT950014

IMMUNOLOGY OF LYMPHATIC FILARIASIS T CELL RESPONSIVENESS AND PROTECTIVE ANTIGENS

Period: January 1, 1996 - December 31, 1998

Co-ordinator: UNIVERSITY OF EDINBURGH
Edinburgh, United Kingdom (R. MAIZELS)

Objectives

- To identify protective antigens in lymphatic Filariasis suitable for vaccine use.
- To measure human antibody and T cell responses to these antigens.
- To clone and express major T cell immunogens as recombinants.
- To express selected recombinant filarial antigens for vaccine tests in animals.
- To enhance expertise and research resources of endemic count scientists.

Activities

* Identification of subjects exposed to Filariasis transmission but show evident immunity.
* Measurement of antibody responses to a range of recombinant antigens.
* Identification and cloning of filarial antigens which most strongly stimulate T cells.
* Analysis of different geographic strains of Brugia malayi.
* Expression of filarial T cell for vaccine testing in rodent models.

Expected outcome

⇒ Identification of a panel of immune individuals whose serological and lymphocyte reactivities will define key in immunity.

⇒ Identification of the major T cell antigens in lymphatic Filariasis, and their cloning and expression in a variety of vectors.

⇒ Evaluation of potential vaccine antigens against lymphatic Filariasis in rodent model systems.

Contract number IC18CT950014

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Contract number ERBIC18CT950026

VACCINATION AGAINST FILARIAL INFECTIONS/USE OF *LOA LOA* A MURINE MODEL FOR IDENTIFICATION OF PROTECTIVE MECHANISMS AND ANTIGENS

Period: January 1, 1996 - June 30, 1997

Co-ordinator: MUSEUM NATIONAL D'HISTOIRE NATURELLE
Paris, France (ODILE BAIN)

Objectives

* To determine the mechanisms of protection induced by irradiated infective larvae of *Loa loa* in monkey and of *Litomosoides sigmodontis* in laboratory mice.

* To identify the mechanisms of natural protection against microfilariae of *L. sigmodontis* in laboratory mice and cotton rats.

* To identify and clone antigens associated with protection, for incorporation, into live single dose oral recombinant vaccines.

Activities

* Spleen T-cell and antibody responses will be measured in *L. simodontis* vaccinated mice and controls, in the presence of antigens derived from microfilariae, L3 and adult worms. Knock-out mice and cytokine depletion with specific monoclonal antibodies will be used. The pattern of antigen recognition will be investigated by Western blot and radio-immuno-precipitation.

* A similar investigation will be performed with *Loa loa* mandrills.

* Survival and migration of *Loa loa* L3 in vaccinated and control mice, and the associated pathology will be studied.

* The pattern of microfilaraemia and the immune response of mice and cotton rats infected with normal *L. sigmodontis* L3 will be investigated, as in 1. Modulatory effects of cytokines will also be investigated.

* mRNA from *L. sigmodontis* and *Loa loa* infective larvae will be isolated for production of cDNA libraries. cDNA clones identified in the primary screens will be sub-cloned in high expression vectors for production of the corresponding recombinant antigens.

* Screening reagents (rabbit antisera) from *L. loa* and *L. sigmodontis* stages (adult worms, microfilariae, infective larvae, fourth stage larvae) will be prepared. Antisera raised against fractionated native and recombinant antigens will be used to localise the molecules in the parasites.

Contract number ERBIC18CT950026

Expected outcome

1) Determination of the relative role of Th1 versus Th2 responses in the development of protective immunity following immunisation of mice with irradiated infective larvae of *L. sigmodontis*.

2) Comparison with the *Loa loa* specific immune responses in mandrills following a similar procedure of immunisation with irradiated larvae.

3) Comparison with natural protection against microfilariae of *L. sigmodontis* in mice and cotton rats.

4) Identification and production of at least some antigens associated with protection.

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Contract number IC18*CT970245

IMMUNOLOGY OF LYMPHATIC FILARIASIS

Period: January 1, 1998 - December 31, 1999

Co-ordinator: UNIVERSITY OF EDINBURGH,
Inst. of Cell, Animal and Population Biology,
Edinburgh, United Kingdom (R. MAIZELS)

Objectives

- To establish the relationship between immune response to filarial parasite antigens and the length of exposure to transmission.
- To identify major antigens from the infective L3 stage of *Brugia malayi*, as defined by reactivity with human immune sera and T cells.
- To identify the major antigens from the microfilarial (Mf) stage, and to test for antigenic variants of these antigens.

Activities

The immunology of exposure to and infection with lymphatic filarial parasites, with respect to T cell subsets and stage-specific antigens, will be assessed. The first aspect will be the relationship between exposure to filarial transmission and the evolution of immunomodulated responses, evident in a strongly TH2 biased, TH1 down-regulated profile. Patients subject to lifelong transmission of filariasis will be compared with immigrants into the same area whose exposure is limited to only 4 or 8 years. The progression of the T cell subset bias, and the development of antigen-specific unresponsiveness seen in filariasis, will be monitored. An important question is whether this subset bias relates primarily to the adult stage, or whether other stages are similarly involved.

Secondly, prominent antigens from L3 and Mf stages will be isolated and cloned for functional analyses. The importance of such antigens in mediating protection against disease can be directly assessed using sera and T cells from the study populations; their biological role in the host-parasite relationship may also be deduced from database sequence similarities and the opportunity to discover new antigens from immature parasites through the new Filarial Genome Project.

A number of other biological questions which underpin filarial disease and its cure will also be addressed. Preliminary evidence for antigenic polymorphism in the Mf sheath antigens will be pursued, using different isolates and species of filarial parasite. The interaction between the anti-filarial drug, DEC, and Mf antigens will be studied. The role of non-CD4 T cells, which may prejudice the immune response towards the TH2 pathway from a very early stage, will be examined.

Contract number IC18*CT970245

Expected outcome

⇒ An understanding of stage-specific immune responses in pathology and immunity.

⇒ Molecular data on sequence and polymorphism of Mf sheath antigens.

⇒ Sequence and profile of immune recognition of L3 antigens.

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Contract number IC18*CT970257

BRANCROFTIAN FILARIASIS: THE INTERRELATIONSHIP BETWEEN TRANSMISSION, INFECTION, HOST RESPONSE AND CLINICAL MANIFESTATIONS IN ENDEMIC COMMUNITIES BEFORE AND AFTER INTERVENTION

Period: September 1, 1997 - August 31, 2000

Co-ordinator: THE DANISH BILHARZIASIS LABORATORY,
Charlottenlund, Denmark (P.E. SIMONSEN)

Objectives

- To assess and analyse the effect of different levels of transmission on the age profiles of infection, disease and host immune response in bancroftian filariasis, and to analyse the interrelationship between these.

- To assess and analyse the heterogeneity of infection, disease and host response patterns within endemic communities, especially the relationships between infection status of children and that of their parents and the effect of exposure at household level on between-host variability in infection and disease levels.

- To assess and analyse the effect of control measures on transmission, infection, disease and host immune responses in communities with different levels of endemicity, and to analyse the cost-effectiveness of these measures.

Activities

The field part of the project will be carried out in Tanga Region of Tanzania and in Kwale District of Kenya, where the partner members have intensive experience from studies on the epidemiology, transmission, chemotherapy and immunology of bancroftian filariasis. Basically, the project will comprise of a series of cross sectional parasitological, clinical and immunological investigations accompanied by intensive longitudinal entomological and clinical surveillance at village and household level in selected high and low prevalence communities. Mass chemotherapy with DEC will be introduced after the first baseline year, while the same type of investigations continue.

Data from individually designed sub-studies will first be analysed and documented separately. However, the design of the project provides a unique opportunity for evaluating the forms of the functional relationships occurring between exposure, infection and disease dynamics, and host immune responses, both at community and individual level. These relationships will be studied via a combination of mathematical models and more advanced statistically-based analyses.

Contract number IC18*CT970257

Expected outcome

The project will address some important unanswered questions related to bancroftian filariasis epidemiology and control, in particular the following:

⇒ What is the role of transmission intensity in shaping the age profile patterns of infection, disease and host immune responses seen in endemic areas, and what roles do transmission intensity and various host immune responses play in resistance mechanisms and pathological processes?

⇒ What is the role of individual exposure intensity in determining the heterogeneity of infection, disease and host response patterns seen within the endemic communities? And what role do parents infection status play for the infection status and immune responses seen in their children?

⇒ What effect do parasite intervention measures have on transmission, infection, disease and host immune responses in communities with different levels of endemicity, and especially at what level of microfilaraemia in the human population will transmission cease? And how will cost-effectiveness of intervention measures differ in communities with high and low endemicity?

Partners

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Contract number ERBTS3*CT910037

CHARACTERIZATION AND UTILIZATION OF TWO NEW FILARIAL MODELS FOR INVESTIGATION OF IMMUNITY, CHEMOTHERAPY AND PATHOLOGY

Period: March 1, 1992 - February 28, 1995

Co-ordinator: MUSEUM NAT. D'HISTOIRE NATURELLE/CNRS-URA 114, LABO DE BIOLOGIE PARASITAIRE, PROSTISTOLOGIE, Paris, France (O. BAIN)

Objectives

The objective of this proposal is to utilize two new models for filariasis: *Litomosoides sigmodontis* in the white mouse and *Monanema martini* dermal microfilaria in a species of murine, in order to:

- ♦ investigate the immunological and genetic mechanisms involved in the pathology of filariasis and of the both natural and acquired resistance to infection.
- ♦ evaluate the action of antifilarial drugs, for example Ivermectin and macrofilaricides, on both the parasite and the host.

Activities

The French partner (Paris) has the two models available and is specialized in parasite biology, morphology and pathological anatomy. This laboratory has contributed in the following ways:

* Definition of the parasitological findings for the two models.
* Vaccination with irradiated L3s, recombinant antigens from experimental filaria and their homologues.
* The effect of macrofilaricides on the parasites and the host, including pathology and immunology.
* Pathological investigations using ophthalmology and pathological anatomy.

The UK partner (Cambridge) possesses great experience in the characterization of parasite antigens and immune responses. The work plan was as follows:

* The analysis of the humoral and cellular response stimulated by *L. sigmodontis* in different strains of mice, for example in Balb/c and Balb/k (susceptible) or B10 (resistant).
* The production of recombinant antigens of *O. volvulus* from different life cycles for vaccination experiments.
* The analysis of the immune response following attempts at vaccination.
* The estimation of the role of T cells (Th1 and Th2) and the production of cytokines. Some of this work will be carried out using the rodent *Lenmiscomys striatus*.

The German partner (Tübingen) will study the mode of action of Ivermectin in the two animal models. Different protocols will be used and the immune response and pathology analyzed in each case.

The Nigerian partner (Enugu) will analyze problems of drug distribution in the host and the parasite as well as the problem of drug resistance, including the pharmacokinetics, detoxicification mechanisms and enzymology.

Expected outcome

It is expected that this investigation will lead to progress in understanding:

⇒ The mechanisms of resistance following vaccination.
⇒ The mechanism of action of the drugs used and the role of the immune response.
⇒ The mechanisms important for the development of ocular and dermal pathology, as well as consequences for the choice of the appropriate drug regime.

Contract number ERBTS3*CT910037

Results

⇒ Resistance, or tolerance, in the natural hosts: reduction of the ratio filariae recovered: infective larvae inoculated was obtained by multi-inoculation of homologous L3, and inoculation of heterologous L3 (*Acanthocheilonema vite/M. martini*). Three other procedures were started: inoculation of offspring from infected and uninfected parents; long term administration of cytokines to infected rodents with an osmotic pump; antifilaricidal drugs.

⇒ Biology and immunology in the *L. sigmondontis*-mouse system: ten strains of inbred mice were sub-cutaneously inoculated with *L. sigmodontis* L3. The Balb/c is the most susceptible (100% mice with adult filaria, 47% with blood microfilariae 2 months p. I.). The BIOD2 is almost totally resistant. The proportion of larvae which develop was similar in the two strains during the first month p.i., but since the tenth day the filarial growth was reduced in the BIOD2.

⇒ Molecular cloning, description and properties of five *O. volvulus* recombinant antigens: Ov 1.9 (16000Mr), Ov 2.5 (50,000Mr), Ov 3.11 (42,000Mr), Ov HSP and Ov GST.

⇒ Vaccination trials in *L. sigmodontis*-mouse model: 83% of reduction of the recovery rate was obtained with an irradiated L3 procedure. Use of a Triton insoluble fraction of *L. sigmodontis* resulted in a facilitating effect. Among the recombinant antigens tested, Ov 3.11 showed a slight sterilizing effect. Experiments of poly-vaccination using *Salmonella typhimurium* with Ov GST or HSp were started.

⇒ Pathology: Onchocerca-like ocular lesions induced by *Monanema martini* were confirmed by an ophthalmological study of 49 *Lemniscomys striatus* and 26 *Arvicanthis niloticus*, the chorio-retinal atrophy and the punctate keratitis are respectively predominant is these two murine hosts, which also represent two biological systems: high microfiladermia and adult worm with long life in the first murine species, low microfiladermia and adult worm with short life in the second. Histology showed that the chorioretinal atrophy corresponds to the destruction of the pigmentary epithelium and sensorial retina, which evokes a degenerative process originating from choroidal vascularization.

⇒ Drug treatments and side effects in *M. martini-L. striatus*: among the 14 drugs tested, one of the four benzimidazoles used, UMF 289, appeared very efficient (micro-and macrofilaricidal) and with less acute side-effects as shown by the histopathological analysis. *M. martini* microfilariae, which are resistant to ivermectin *in vitro* and *in vivo*, were also resistant to moxydectin.

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Contract number: ERBTS3*CT920056

CAN CATTLE PROTECT MAN FROM ONCHOCERCIASIS?: REDUCTION OF SIMULIUM VECTORIAL CAPACITY AND CROSSREACTING IMMUNITY AS FACTORS OF ZOOPROPHYLAXIS

Period: April 1, 1992 - March 31, 1996

Co-ordinator: UNIVERSITÄT HOHENHEIM, DEPARTMENT OF PARASITOLOGY, Stüttgart, Germany (A. RENZ)

Objectives

Despite an extremely high *Simulium damnosum s.l.* biting rate on man (up to 150.000 bites per year at the riverbank) and intensive *Onchocerca* transmission potentials (60.000 L3 *O. ochengi* and 2.000 L3 *O. volvulus* per man and year), human onchocerciasis was found to be rare in a cattle-farming highland area of Cameroon. The concept of zooprophylaxis was studied with a view to:

- Assessing the reduction of the vectorial capacity of *Simulium* populations as a result of blood meals on non-human hosts.
- Establishing the degree of cross-transmission of invasive L3s of various strains and species of *Onchocerca* parasites between humans and animals, particularly cattle.
- Determining whether antigenic cross-reactivity exists between *O. volvulus* and *O. ochengi* and whether exposure to *O. ochengi* affords a degree of protection against *O. volvulus* in man.
- Utilizing the *O. ochengi* cattle model as a system for testing potential vaccines for human onchocerciasis.

Strategies for local onchocerciasis control can be developed integrating live stock production and socio-economic development in rural areas. The proof of concomitant homologous and cross-reacting heterologous immunity in human and bovine onchocerciasis would support the development of a vaccine against human onchocerciasis.

Activities

Activities have been broadly divided into two categories - studies of the epidemiology (Germany, Cameroon and Nigeria) and the immunology (Glasgow). The epidemiological topics addressed included: (i) the life cycles of *Simulium*-transmitted animal filariae, using microfilaria isolated from fresh hides injected intrathoracically into neonate *Simulium* flies; (ii) whether L3 invade unsuitable hosts, by assessing the readiness of the various species of filariae to leave their vector during a blood meal; (iii) the prevalence of human and animal onchocerciasis in selected villages in Cameroon and Nigeria; (iv) *Simulium* biting rates and *Onchocerca* transmission potentials on man and cattle; (v) investigations on the biology and epizootiology of bovine onchocerciasis, *O. ochengi* in particular; (vi) cross-immunization experiments in cattle, using *O. volvulus* larvae as a live vaccine against a challenge infection by *O. ochengi* L3.

Immunological topics covered: (i) *Onchocerca*-antigen recognition by human and bovine sera; (ii) comparison of surface and excretory-secretory (E-S) antigens of *O. volvulus* and *O. ochengi* using biochemical preparative techniques followed by SDS-PAGE and isoelectric focusing; (iii)antigenic analysis of radiolabelled molecules for antigenicity and cross-reactivity and (iv) investigation of lymphocyte responses.

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Expected outcome

The results of this study will enable:

⇒ The national health authorities in Cameroon and Nigeria to formulate control strategies and to assist local public health authorities in the control of onchocerciasis - these results could have a direct impact on rural development planning.

⇒ An evaluation to be made of the epidemiology and intensity of disease transmission before the mass-distribution of ivermectin, which is expected to strengthen the effects of zooprophylaxis by reducing the proportion of *O. volvulus* larvae in the man-biting files.

⇒ An assessment to be made of the importance of cross-reactive immunity and the possibility of vaccine development.

Results

Quantitative aspects of the transmission dynamics of *Onchocerca volvulus*, *O. ochengi* and *O. ramachandrini* have been studied in savanna and rain-forest areas of Cameroon and Northern Nigeria. The proportion of non-*O. volvulus* infective larvae (L3) in man-biting *Simulium damnosum* s.l. flies in the Sudan savanna (prior to the now starting ivermectin campaigns) varied from 20-60% and consisted mainly of *O. ochengi* during the dry season, when nomadic cattle gather along the perennial rivers, with an increasing proportion of *O. ramachandrini* (former Type D sensu stricto, adult worms in warthogs) during the rainy season. In the Adamaoua highland, where cattle outnumber the human population by a factor of 5 to 20, over 90% of all infective larvae were *O. ochengi*, but warthogs and *O. ramachandrini* are absent. *O. volvulus* L3 leave their vector flies during a blood meal on cattle in similar proportions as do *O. ochengi* L3 (ca 60 to 80% of L3). The same escape seems to occur if L3 of *O. ochengi* are transmitted on man by boo-anthropophilic flies. This is a prerequisite for a naturally occurring crossreactive immunization.

The homology of *O. volvulus* and *O. ochengi* gravid female worm antigen and their recognition by sera of human onchocerciasis patients from villages exposed to various amounts of *O. Ochengi* co-transmission was studied by one- and two dimensional gel electrophoresis. There was a high degree of homology in the protein composition of the two filarial species and a marked crossreactivity in the humoral recognition of antigens. Patients groups from the savanna (with high microfilarial density in the skin) recognized low molecular weight antigens of both filarial species more strongly than patients from the highland (which had few microfilaria or were skin-negative). However, antibodies from sera of highland patients bound to *O. ochengi* rather than to *O. volvulus* antigens. These patients also showed a pronounced IgG3-reactivity to *O. ochengi* antigens indicating that IgG3 might be important in acquired immunity to onchocerciasis.

Excretory/secretory (E-S) and surface antigens are likely to represent potential targets for protective immune responses. Infective larvae of *O. volvulus* and *O. ochengi* have therefore been subjected to a variety of intrinsic (biosynthetic) and extrinsic radiolabelling procedures in order to assist E-S. antigen analysis by two dimensional electrophoresis. In spite of much effort, only the extrinsic procedures (iodination) resulted in a degree of radiolabelling applicable to analysis and only with E-S Iodinated *O. volvulus* L3 E-S appear to contain four major products with molecular weights of 180, 140, 55 and 51 kD and *O. ochengi* L3 appear to contain at least three of these.

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Highly sensitive and specific ELISAs have been developed for the measurement of bovine IgM, IgG1 and IgG2 antibodies to parasite molecules. These assays demonstrate that infection is generally quickly associated with elevated IgM and IgG1 levels to parasite antigen. IgG2 levels appear to be much less affected. Conversely, we have obtained some preliminary evidence that IgG2 levels may rise following our successful "vaccination" of cattle against *O. ochengi* with living *O. volvulus* infective larvae. As the vaccination result is of potential importance, we are currently investigating this interesting possibility further using both ELISA and Western Blotting to target individual antigens being recognized.

A sandwich ELISA for detection of phosphorylcholine-containing circulating antigen has been applied to cattle from Cameroon infected with *O. ochengi*. Preliminary observations suggest that it can successfully detect infection and hence it may be of some value in the cases where the presence of infection is uncertain.

An heterologous cross-immunization experiment was carried out with the aim of immunizing calves with live infective larvae of *O. volvulus* against a challenge infection by *O. ochengi* L3. In the 10 naive, non-vaccinated calves, 26 worms (22 female and 4 male worms) had developed to the adult stage and were found in 22 palpable nodules (26 of 254 L3 larvae injected = 10% development rate). In the vaccinated group (10 animals, 545 to 800 live *O. volvulus* L3 s.c. per calf), only 2 nodules containing 2 females but no males were recovered (2 of 254 *O. ochengi* L3 injected = 1.5%). This experiment showed that the human parasite afforded some degree of protection against a challenge infection in cattle, much in the same way as the bovine parasite protects the human population. It is the first successful vaccination in a nodule-forming *Onchocerca*-species using a live vaccine in its natural host.

In conclusion, our results strongly corroborate the hypothesis of cross-protection between the bovine and human parasite and confirmed that *O. volvulus* is indeed unable to establish patent infection in the bovine host. The now-starting mass-distribution of ivermectin in Africa is likely to enhance these effects of cross-immunization by reducing the proportion of infective L3 of *O. volvulus* in the anthropozoophilic Simulium vector flies. A mathematical model is presently being developed to describe the quantitative effects of zooprophylaxis and cross-transmission on the epidemiology or human onchocerciasis.

Contract number: ERBTS3*CT920056

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Contract number: ERBTS3*CT920058

IMMUNE RESPONSES IN ONCHOCERCIASIS: STUDIES WITH MOLECULAR PROBES IN PATIENTS AND AN ANIMAL MODEL

Period: October 1, 1992 - September 30, 1995

Co-ordinator: UNIVERSITY OF SALFORD, DEPARTMENT OF BIOLOGICAL SCIENCES, Salford, United Kingdom (J.E. BRADLEY)

Objectives

The major objective of this project were to produce recombinant proteins of the parasitic nematode *Onchocerca volvulus* for the development of specific serodiagnostic tests and candidate vaccines. A parallel approach involved the use of the rodent filarial model system *A. vitae* in the Jird and the production of recombinant proteins from this parasite with the objective of performing protection experiments.

Results

A) Isolation and characterisation of recombinant proteins.

Many recombinant antigens have been isolated and expressed as part of this project. Most have been previously reported. Recent work has focused on the cloning, expression and purification of the full length molecule corresponding to a previously described 20kDa molecule Ov 20. This has now been achieved using the pET 15 expression system and it is now possible to express this molecule at a level of 100mg/L. This has allowed us to perform a number of experiments to determine the structure and function of this molecule.

It has been determined using a fluorescence binding assay that Ov20 binds retinoids strongly with a dissociation constant of 0.085μM. It also binds other fatty acids but not as strongly as retinol (dissociation constant 0.97μM). This is in contrast to a recently described fatty acid binding protein; the polyprotein allergen from Ascaris. The structure was shown to be 60% alpha helix by circular dichroism and using structure prediction programmes it was also predicted to be predominantly alpha helical and formed a coiled coil which is highly unusual for such proteins. Thus, it appears that Ov20 represents a novel retinoid binding protein with a structure quite unlike any so far described.

Retinoid binding proteins have been isolated by protein purification from *O. volvulus* in the past and based on size (19kD) may be identical to Ov20. This molecule was shown to be able to bind ivermectin. As Ov20 represents such an unusual molecule it may be an important target for chemotherapy.

B) Serodiagnosis and seroepidemiology

Collection of well characterised patient sera from the hyperendemic area of the Lekie region of Cameroon has been described in earlier reports. As has the utilisation of various recombinant antigens for serodiagnosis. The serodiagnostic part of the project has largely been completed. The use of a cocktail of three recombinant antigens (Ov10, Ov11 and Ov29) to detect antibody in patients sera work with a high degree of specificity and sensitivity. The addition of a fourth antigen to the cocktail (Ov33) was shown to further improve the sensitivity.

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Epidemiological studies using sera from Cameroon in attempts to associate disease status with antigen recognition have, in general, proved fruitless. All the recombinant antigens, isolated as part of this project, have been tested at the isotype level with sera from ophthalmologically characterised onchocerciasis individuals but no correlations were found. Analysis of sera from these individuals by immunoblotting against adult worm antigens has indicated that certain antigens were recognised more frequently by these individuals in comparison to asymptomatic patients.

A further aim of the study was to evaluate the effect of host genetics on the outcome of infection. HLA studies have so far revealed no correlation with any disease state or response to any individual antigen. Expression of novel DPB alleles, however, was found in some individuals from this region.

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Contract number ERBTS3*CT920096

PROTEIN COMPONENTS OF CHEMORECEPTOR ORGANELLES AND OF THE CUTICLE OF NEMATODES: IDENTIFICATION AND CLONING OF THE GENES, PRODUCTION AS RECOMBINANT PROTEINS AND ANALYSIS OF THE IMMUNE RESPONSE ELICITED

Period: January 1, 1993 - December 31, 1995

Co-ordinator: CENTRO INTERUNIV. RICERC. PAESE IN VIA DI SVILLUPO
Napoli, Italy (P. BAZZICALUPO)

Objectives

- Increase our knowledge and understanding, at the cellular and molecular level, of the ontogenesis, structure and physiology of chemoreception and of the cuticle of nematodes.
- Clone and study parasite molecules, components of the chemoreception system and of the cuticle, as potential target for drug and/or vaccine development.
- Study the immune response toward molecularly defined antigens of nematodes.
- Perform pilot epidemiological studies to identify, in the Northern region of Morocco, special communities or situations were nematode infections are more frequent and/or severe.
- Train scientists from European and developing countries in the field of biotechnology as applied to the control of nematode infections.

Activities and results

Chemoreception

We have completely characterized a chemoreception mutant identifying a new gene dyf-1 and have shown that it affects the structure of the amphids, the main chemosensory organs of nematodes.

Avoidance mutants

We have designed a completely new test for the chemical avoidance response. The test is relatively easy to perform, fast and unambiguous and has enabled us to screen over 150 different chemicals for the ability to trigger the avoidance reflex in *C. elegans*. Among the newly identified repellents are cupper ions, quinine and other antimalarial drugs. Using the assay described above, we have isolated 13 new mutants unable to avoid quinine. Some of these mutant are particularly interesting in that they apparently have normal cilia and amphidial channels. Two of these mutants do not complement and define a new gene qui-1 which we have mapped genetically on chromosome IV near the gene tra-2. The mutants fail to avoid quinine but are still able to avoid other repellents, including cupper ions and garlic extracts. This finding, together with the apparent normal architecture of the cilia, suggests that the two mutations are likely to affect the receptors directly and not the general functioning of chemosensory neurons.

Cuticle

We have identified and cloned three homologues of cut-1 in *Ascaris lumbricoides* and at least two in *Brugia pahangi*. Southern blots with conserved regions have shown that genes homologous to cut-1 are conserved in all other nematode species tried.

Together with several cut-1 like genes which have been discovered in *C. elegans* by the genome sequencing project they represent a new gene family with an important role in

making up the protective layers of nematodes. One Ascaris gene has been studied more completely and the whole genomic and cDNA sequences determined. The homology with *C. elegans* cut-1 is higher than 85% as the amino acid level and like in *C. elegans* the protein begins with a signal peptide.

Recombinant Ascaris CUT-1 has been obtained from *E. coli* expression vectors and used to raise specific antisera in rabbits. Immuno-electron-microscopy has been used to localize CUT-1 and CUT-2 epitopes in nematode cuticles. Determination of the genomic sequences and identification of cDNA clones from *Brugia pahangy* are in progress in Glasgow.

Using recombinant CUT-2 produced in *E. coli* we have studied CUT-2 cross-linking *in vitro* and demonstrated the importance of hydrophobic interactions in this process, which occurs during cuticle assembly *in vivo*, and involves the formation of dityrosine bridges between different CUT-2 molecules.

Immune response to gp 30 and filarial antigens

We performed a series of experiments to establish the cellular and immune response of mice to native gp30 and to a synthetic gp30 peptide which corresponds to a T cell epitope. A variety of immunization protocols were used:

⇒ mice immunized and boosted with L3 of *Brugia pahangi*;
⇒ mice immunized sub-cutaneously with 100 micrograms of the synthetic gp30 peptide or with 100 micrograms of native Brugia antigen;
⇒ mice infected with adult *B. pahangi* by transplantation into the peritoneal cavity;
⇒ mice immunized by footpad injection with the peptide and or the adjuvant alone.

Sera and lymphocytes from these animals were then analyzed for presence of specific antibodies, for proliferation for cytokine production. In general, peptide immunized animals had no detectable antibody response to Brugia antigen, while animals immunized with the native antigen or with worms did. Similarly, lymphocytes from native antigen immunized animals proliferated in response to Brugia antigen but not to peptide while cells from peptide immunized animals did not proliferate in response to native antigen but there was a modest stimulation with peptide. Finally no differences were observed in the expression of any cytokine mRNA from lymph nodes of peptide immunized animals compared to adjuvant only controls.

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Contract number: ERBTS3*CT930235

GENETIC VARIATION IN *ONCHOCERCA VOLVULUS* FROM CAMEROON, TANZANIA AND SIERRA LEONE

Period: January 1, 1994 - June 30, 1996

Co-ordinator: KONINKLIJK INSTITUUT VOOR DE TROPEN, DEPT OF BIOMEDICAL RESEARCH Amsterdam, The Netherlands (L. OSKAM)

Objectives

- To investigate the genetic diversity within *O. volvulus* using molecular markers.
- To assess the role of strain variation in the epidemiology of onchocerciasis.

Activities

* We collected *O. volvulus* samples from foci from different biotopes within different endemicities and with different parasitological, clinical, and ophthalmological manifestations.
* We investigated the genetic variation within *O. volvulus* from Cameroon, Tanzania and Sierra Leone using molecular biological techniques such as microsatellite PCR, Southern blotting and hybridization and nucleotide sequencing.

Results

Epidemiology

In Cameroon *O. volvulus* samples were collected in five villages located in three different biotopes, namely savannah, forest and savannah-forest mosaic. In all villages prevalence rates were well above 50%. There appeared to be no difference in blindness rates between the three biotopes.

In Tanzania samples were collected in eight villages located in four foci: 3 savannah foci and one altitude forest focus. In three out of four foci we found a high percentage of the people (9-18%) presenting with onchocerciasis related blindness. To our knowledge this is the first description of the extent of blinding onchocerciasis in East Africa.

Random amplified polymorphic DNA analysis (RAPD)

We tried to develop a RAPD PCR for the detection of genetic differences between *O. volvulus* isolates. We found that this technique is not suitable for the analysis of *O. volvulus* DNA because of the presence of traces of human DNA present in the nodule tissue which clings to the worms during isolation. This lead to extremely polymorphic patterns. Moreover, the patterns were highly dependent on the amount of input DNA.

Nucleotide sequence analysis

From a selection of samples two different parts of the genome were cloned and sequenced.

1. Internal Transcribed Spacer (ITS) This area comprises the intergenic region between the 18S and 5.8S rRNA genes. It is known that this is an area with a high degree of sequence variation. We found that in *O. volvulus* the sequence of the ITS was highly conserved between different isolates.
2. 150 base pair repeat The 150 bp repeat of *O. volvulus* is present at approximately 4500 copies per haploid genome. This makes this repeat an ideal target for PCR. Nucleotide sequence has shown that there is sequence variation within the 150 bp repeat. From the sequence analysis we performed we concluded that there are differences, but that these are not systematic differences, making it impossible to develop specific probes or primers.

PCR and Southern hybridization using O. volvulus and "non-blinding" strain specific probes

Based on the nucleotide sequence of the 150 bp repeat, two oligonucleotide probes have been identified in the past: OVS-2 being specific for *O. volvulus* and OVF for "non-blinding" *O. volvulus*.

We found that probe OVS-2 did not react with 19/99 (19%) of the PCR amplified DNA isolates of *O. volvulus*. All these isolates were from outside the Onchocerciasis Control Programme (OCP) area for which the probes were originally designed.

We found that none of the samples from Tanzania reacted with probe OVF. This corroborates the finding described in "epidemiology" that blinding onchocerciasis is common in Tanzania. In Cameroon the situation was less clear. All 4 isolates from the Savannah forest mosaic that were tested reacted with probe OVF despite the fact that two were isolated from blind people. In the forest area, 90% of the samples reacted with probe OVF.

We conclude that, at least outside the OCP area, one has to be cautious with the interpretation of results obtained with probes OVS-2 and OVF.

Microsatellite PCR

Microsatellites are short (2-5bp) tandemly repeated sequences that have been shown to be useful as polymorphic markers for populations and individuals. We screened an *O. volvulus* genomic library for the presence of microsatellite sequences and identified one locus consisting of a (CA) 30 repeated sequence containing one insertion and 5 point mutations. Based on this sequence, primers were chosen and a PCR amplification reaction was developed and optimized.

With this PCR we were able to identify 7 different alleles at this locus, namely A, B, B', C1, C2, C3 and D. When comparing isolates from Sierra Leone, Cameroon and Tanzania, we found that the isolates from Sierra Leone showed almost exclusively the A allele, the Cameroon isolates showed A, B and B' and the isolates from Tanzania showed all seven alleles, thereby being the most heterogenous population.

We conclude that microsatellite PCR is a suitable technique for the detection of genetic variation between *O. volvulus* isolates. However, it is at the moment impossible to correlate the presence of certain alleles with clinical findings. In order to make this possible, we will have to develop a set of microsatellite PCRs at different heterogenous loci.

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Contract number ERBTS3*CT940271

ANTI-OXIDANT ENZYMES IN FILARIAL NEMATODES: THEIR ROLE IN DEFENCE AND PERSISTENCE

Period: July 1, 1993 - June 30, 1996

Co-ordinator: BERNHARD NOCHT INSTITUTE FOR TROPICAL MEDICINE, Hamburg, Germany (K. HENKLE)

Objectives

- To characterize the anti-oxidant enzymes, glutathione peroxidase and superoxide dismutase, in *O. volvulus* and *B. malayi*, and to define the stage-specificity of expression and secretion of these enzymes *in vivo* and *in vitro*.
- To assess the role of anti-oxidant enzymes in different stages of filariae in the defence against mediators of cytotoxicity and to biochemically define the damage caused by oxidants.
- To train scientists from Banaras Hindu University in techniques of molecular biology and biochemistry, and to integrate and co-ordinate the efforts of independent laboratories working in closely related subject areas.
- To assay the immune response to anti-oxidant enzymes in human filariasis patients, and in particular the effect of antibodies which neutralize parasite enzyme activity.

Results and activities

* The glutathione peroxidase has been extensively characterized in *B. malayi*, including its structure, activities, localization, stage-specificity and secretion. A functional counterpart in *O. volvulus* has not been identified, although an enzyme with overlapping function and a very similar localization profile, the glutathione S-transferase I, has been characterized.
* Two distinct CuZn SODs have been characterized in *O. volvulus* and *B. pahangi*. The structure, function, localization, stage-specificity and secretion of these two enzymes in both parasitic nematodes has been examined.
* The characterization of the "extracellular" form of the CuZn SODs in both *O. volvulus* and *B. malayi* provides evidence that this enzyme is involved in the defense against host superoxide anion radicals since it was found to be secreted in *in vitro* and *in vivo* studies.
* To assess the role of anti-oxidant enzymes in different stages of filariae in the defence against oxidative stress and to biochemically define the damage caused by oxidants, *in vitro* studies were performed with *B. malayi*. The sensitivity of microfilariae and adult *B. malayi* to hydrogen peroxide (H_2O_2) was assessed and both stages were found to be surprisingly resistant to peroxide stress. Microfilariae showed an impaired viability at concentrations at which adult worms were unaffected, correlating with the concentrations of the antioxidant enzymes in these two stages.

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* The rate of H_2O_2 consumption by parasites was determined, and showed that adult worms metabolised it at a rate 24 X faster than microfilariae. Assessment of enzyme activities in parasite extracts demonstrated that H_2O_2 metabolism was affected principally by catalase activity, which was elevated in adult worms relative to microfilariae. Cytochrome c peroxidase activity was also detected, and was roughly equivalent in both stages. Glutathione peroxidase and NADH/NADPH-dependent consumption of H_2O_2 were absent, and the rate of non-enzymatic reduction of H_2O_2 coupled to glutathione oxidation did not contribute significantly to metabolism. Glutathione reductase activity and total glutathione content were equivalent in adults and microfilariae.
* It was determined that *B. malayi* is much more resistant to H_2O_2 than other filarial species examined to date, and can effectively metabolize levels in excess of those potentially generated by activated leucocytes.
* Dr. Henkle-Dührsen has visited Dr. Selkirk in London and Dr. Rathaur in Varanasi to discuss results and coordinate future experiments. Dr. Selkirk will visit Dr. Henkle-Dührsen and Dr. Rathaur late in 1996 to analyze, integrate and summarize results from each partner.
* The human immune responses of Indian patients infected with *W. bancrofti* have been examined with the recombinant glutathione peroxidase and superoxide disumutase enzymes. Studies to detect antibody reagents which neutralize enzymatic activity are in progress.
* Dr. Suman Misra from Varanasi spent 7 weeks in 1995 at the Bernhard Nocht Institute in Hamburg for training in molecular biology methods, as well as in the production and purification of recombinant proteins. Dr. Sushma Rathaur spent 6 months in 1996 at the Bernhard Nocht Institute in Hamburg to learn advanced molecular biology methods.

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Contract number ERBIC18CT950017

ISOLATION, CHARACTERISATION AND IMMUNOLOGICAL EVALUATION OF RECOMBINANT VACCINES FOR FILARIAL PARASITES

Period: January 1, 1996 - December 31, 1998

Co-ordinator: SALFORD UNIVERSITY
Salford, United Kingdom (J.BRADLEY)

Objectives

- Development of candidate vaccines against parasitic filarial infections, in particular *Onchocerca volvulus.*
- To identify, isolate and express antigens that are specific to the larval stages of these parasites.
- To analyse of the immune responses of humans exposed to *O.volvulus* and animals infected with *Acanthoche lonema viteae* to these antigens.
- To assess the protective capacity of these antigens in a rodent filarial model system.

Activities

* Production of quantities of L3 and L4 larval stages of the parasites *Onchocerca volvulus* and *Acanthocheilonema viteae.*
* Identification and characterisation of antigens that are specific to the larval stages of the parasite by protein analysis and differential display PCR.
* Cloning and Expression of stage specific antigens from cDNA libraries of each larval stage.
* Characterisation of the cellular and humoral responses to the larval antigens of putatively immune humans exposed to onchocerciasis transmisson.
* Evaluation of the protective capacity of the recombinant larval antigens in a rodent filarial model system.

Expected outcome

⇒ The identification of novel candidate vaccine antigens for onchocerciasis.

⇒ The identification of the type of immune responses that are protectiveagainst filarial infections in humans and animal models.

Contract number ERBIC18CT950017

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Contract number: ERBTS3*CT920057

IVERMECTIN - FACILITATED IMMUNITY IN ONCHOCERCIASIS

Period: December 1, 1992 - November 30, 1995

Co-ordinator: UNIVERSITY OF TÜBINGEN, INSTITUT FÜR TROPENMEDIZIN, Tübingen, Germany (P.T. SOBOSLAY)

Objectives

- To activate parasite-specific cellular responses of onchocerciasis patients before and after single and repeated doses of ivermectin.
- To characterize antibody responses of onchocerciasis patients before and after ivermectin treatment in terms of isotypes and antigen specificity.
- To characterize and localize immunodominant *Onchocerca volvulus* antigens potentially involved in protection or pathology.
- To examine a possible immuno-dependent regulation of the reproduction of *Onchocerca volvulus.*

Activities and results

In West Africa, control of onchocerciasis relies on both vector eradication and mass treatment campaigns with ivermectin, the drug of choice for onchocerciasis treatment. Humans chronically infected with *O. volvulus* demonstrate not only a prominent production of all subclasses of parasite-specific immunoglobulins but also a depressed cellular reactivity *in vitro* and deficient production of cytokines in response to *O. volvulus*-specific antigenic stimulation. A longitudinal investigation was conducted into the cell-mediated immune responses of onchocerciasis patients after a single-dose treatment with ivermectin. The *in vitro* cellular reactivity to *Onchocerca volvulus* - derived antigens (OvAg) was reduced in untreated patients as compared to controls, and the cellular reactivity to OvAg and bacterial antigens improved up to 14 months post treatment. These results suggested a distinctly improved cellular immunity in human onchocerciasis that was facilitated by single ivermectin therapy. In continuation, the parasite-specific antibody response, cellular reactivity and cytokine production profile was examined in onchocerciasis patients repeatedly treated with ivermectin for several years. Cellular reactivity to OvAg increased after several rounds of ivermectin dosing, and furthermore, proliferative responses were greater in treated microfilaria-free patients than in microfilaria-positive onchocerciasis patients. In amicrofilaridermic patients such reactivity approached the magnitude observed in endemic control individuals. Peripheral blood cells from subclinical amicrofilaridermic patients or exposed endemic controls produced significantly more IL-2 and IFN-γ in response to OvAg than cells from patently infected, mf-positive humans. Titers of parasite specific IgG subclasses continuously declined with repeated treatments, and *Onchocerca volvulus* antigen recognition by circulating antibody isotypes gradually weakened. These results suggest that onchocerciasis-associated immunosuppression is reversible following ivermectin-induced permanent clearance of microfilariae from the skin; and that a vigorous parasite-specific Th1-type reactivity in amicrofilaridermic individuals may contribute to controlling *Onchocerca volvulus* infection.

In order to identify those parasite antigens which may account for the differential cellular responsiveness with distinct states of *Onchocerca volvulus* infection total adult worm-derived OvAg were fractionated into antigen fractions of continuously decreasing molecular weight.

While PBMC from onchocerciasis patients did not respond to any of the OvAg fractions, reactivity of PBMC from exposed endemic controls was depressed to only high molecular weight antigens of Mr 200-30 kDa. However, antigens of Mr<30kDa induced vigorous responsiveness in mf-negative individuals, with highest proliferative responses being observed in response to OvAg of Mr 15-11 kDa. Furthermore, these low molecular weight antigen fractions induced substantial production of IL-2 and IFN-γ in PBMC from endemic controls, but not in those from onchocerciasis patients. In contrast, PBMC from patients clearly produced more IL-10 than did cells from endemic controls. These results suggest that vigorous Th1 type cellular responsiveness encountered in endemic controls is restricted to low molecular weight antigens of *Onchocerca volvulus*, such reactivity is not present in mf-positive individuals. These traits of cellular immunity may contribute to the differential outcome of *Onchocerca volvulus* infection, the manifestation of clinical disease, and may also regulate the build up of acquired immunity in humans.

In onchocerciasis microfilariae play the key role for the transmission of the parasite and the development of pathology, and since microfilariae released from the adult worms accumulate and survive in the host for considerable time, the parasites' reproductive biology and population dynamics are of prime consideration for the control of filariases. The analysis of the reproductive capacity of adult *O. volvulus* has revealed that reproduction occurs in asynchronous cycles, and only a portion of the intra-uterine microfilariae will leave the female worm. The mechanisms that regulate microfilarial production and release seem to be multifactorial; partially or even predominantly regulated by factors and mechanisms intrinsic to the host; e.g. the current state of immunity. In patients without microfilariae in skin biopsies (after ivermectin treatment, or humans with postpatent infection) the reproduction of the parasite continues with regular embryonic and microfilarial development; there was however, a spectacular and unusual accumulation of vital microfilariae in the uteri, microfilariae did not leave the uteri, degenerated and were subsequently resorted. Reduced transmission of the parasite and ivermectin treatment will interfere with the delicately balanced host-parasite equilibrium and with parasite reproduction. Ivermectin-facilitated immunity, as a consequence of repeated therapy, may be of significant relevance to the control of onchocerciasis, immunological alterations in the host which alter the worm population and its reproductive capacity may synergistically help to prevent the clinical manifestations of onchocerciasis.

Conclusions

The severity of chronic disease produced by the parasitic nematode *Onchocerca volvulus* varies widely, ranging from asymptomatic infection to cutaneous involvement and, most severely, to ophthalmologic pathology which may finally cause blindness. At present, onchocerciasis control relies on vector eradication and on mass treatment campaigns with ivermectin. Cellular immunity of onchocerciasis patients underwent substantial alterations following repeated ivermectin treatment indicating that cellular hyporesponsiveness in chronically infected patients is reversible following permanent clearance of microfilariae from the skin. Control strategies which are aimed at reducing aggregation of *O. volvulus* may facilitate the expression and persistence of parasite-specific Th1-type immunity, which may suffice to reduce the serious morbidity of *O. volvulus* infection.

Contract number: ERBTS3*CT920057

Onchocerciasis is the fourth common cause of blindness and inflammatory eye disease. An autoimmune cause for ocular onchocerciasis has been suggested since the identification of a recombinant antigen (Ov39) that shows immunologic cross-reactivity with a human ocular tissue component. The structural similarities between host self-antigens and *Onchocerca volvulus* may thus be involved in expression of autoimmune reactivity and development of autoimmune disease. The tissue distribution of Ov39 correlates with the sites of pathology in onchocerciasis and antibody reactivity could be detected in all persons with onchocerciasis and pathology of the posterior segment of the eye. By immunohistological methods epitopes cross-reactive between parasite Ov39 and human ocular hr44 were localized to the optic nerve, neural retina, retinal pigment epithelium, as well as the epithelial layers of the ciliary body and iris. These observations propose that immunological cross-reactivity contributes to the immunopathogenesis in onchocerciasis, and it is suggested that intraocular presentation of this cross-reactive parasite antigen by microfilariae is essential for the development of disease.

For prevention of onchocercal ocular disease repeated annual treatment with ivermectin is recommended. Repeated treatment for several years effectively reduced ocular microfilariae and achieved a considerable regression of early ocular lesions of the anterior segment of the eye, however, observations also indicated that the most beneficial effects were only achieved, if therapy was conducted regularly. From these observations we conclude, that for the long-term ivermectin-supported control of onchocerciasis, implemented through primary health care activities, the compliance of endemic populations is urgently required.

Contract number: ERBTS3*CT920057

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Contract number ERBTS3*CT920082

EXPLOITATION OF TARGETS FOR CHEMOTHERAPY IN POLYAMINE METABOLISM OF FILARIAL WORMS

Period: February 1, 1993 - October 31, 1996

Co-ordinator: BERNHARD NOCHT INSTITUTE FOR TROPICAL MEDICINE, DEPT OF BIOCHEMICAL PARASITOLOGY, Hamburg, Germany (R.D. WALTER)

Objectives

The project has the following objectives:

- The development of urgently needed, safe and potent drugs against onchocerciasis and lymphatic filariasis.
- The assessment of filaricidal efficacy and selectivity of potential biochemical targets in the polyamine metabolism of filarial worms.
- The strengthening of research capacity in tropical diseases both within the EC and the developing countries, here the consolidation of a standing co-operation started under STD-2 with the University of Benin-City in Nigeria.

Activities

The activities which form part of this project are the assessment and exploitation of peculiarities in the polyamine metabolism of filarial worms with a view to rational drug development. The research project will address the following questions:

* How do filarial worms regulate their polyamine synthesis and content?
* Can the parasite evade toxic levels of polyamines?
* Is the polyamine oxidase a candidate target for chemotherapy?

Results

1. Biosynthesis

   Activity of ornithine decarboxylase (ODC), the initial step in the polyamine synthesis pathway, could not be detected in various parasitic nematodes and it has been suggested that filarial worms do not synthesize putrescine. To obtain a better insight into the polyamine synthesis of nematodes and in order to establish a model for studying polyamine metabolism in culture at the biochemical and molecular level, the free-living nematode *Panagrellus redivivus* was established as an experimental system, which has similarities in overall structure and development to parasitic nematodes. The ornithine decarboxylase gene from *P. redivivus* was isolated, expressed in a bacterial system and both the gene and the recombinant enzyme were characterized at the molecular level. Further attempts to identify the ODC gene in *O. volvulus*, *Brugia pahangi* and *Ascaris suum* by use of PCR analysis as well as screening of genomic and cDNA libraries of these parasitic nematodes with the OCD cDNA from *P. redivivus* as probe were not successful. These results suggest that filarial worms do not depend on their own biosynthesis of purtrescine.

   S-Adenosylmethionine decarboxylase (SAMDC), another rate limiting step in the biosynthesis of polyamines was proposed as potential drug target. The filaricidal efficacy of this target was assessed by treatment of maintained worms with specific enzyme inhibitors, which led to depletion of the polyamine content and to death of the worms.

   To assess the selectivity of the target, the *O. volvulus* SAMDC gene was isolated, expressed in *E. coli* and the recombinant enzyme was analysed on the molecular level.

   The amino acid sequence of the filarial SAMDC shows only a moderate degree of similarity with the mammalian SAMDC, indicating structural differences which are a necessary prerequesite for the synthesis of specific inhibitors.

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2. Polyamine sequestration and N-acetylation

The occurrence and nature of polyamines covalently bound to proteins were investigated in parasitic and free living nematodes. Acid hydrolysis of various fractions of the homogenate of *O. volvulus*, *A. suum* and *P. redivivus*, followed by RP-HPLC analysis clearly confirmed the occurrence of conjugated spermidine and spermine in the soluble and pellet fractions of reproductive tissues, muscles and cuticle. This result is of special interest, because the reversible conjugation of spermidine and spermine could be a process by which the nematodes regulate and control the concentration of "free" polyamines. This could be a mechanism for them to avoid lethal levels of spermine, by sequestering the excessive polyamines. The absence of polyamine N-acetylation, a process which has been reported from *O. volvulus* and *A. suum*, whereas activity of diamine N-acetyltransferase was measured and discovered to be involved in the excretion process of putrescine. In trematodes, a single enzyme has been shown to fulfill the combined function of diamine, polyamine and arylalkylamine acetylation. Investigation of the former nematodes revealed the occurrence of distinct N-acetyltransferases for diamines and arylalkylamines. Based on the absence of polyamine N-acetylation in filarial worms and allied nematodes, it is concluded that the degradation of excess spermine and spermidine proceeds via the interconversion pathway to putrescine, which is acetylated by the diamine N-acetyltransferase prior to excretion.

3. Polyamine oxidase and the polyamine interconversion pathway

In parasitic nematodes, the polyamine oxidase has been shown to be the rate-limiting step in the interconversion pathway. The nematode enzyme differs with respect to substrate and inhibitor specificities from the mammalian counterpart. The parasite specific inhibitor MDL 72145 was considered as a suitable chemical lead for the chemotherapy of filarial parasites, but the chemotherapeutic strategy of spermine accumulation to lethal concentrations failed, since not spermine, but the reaction products of the polyamine oxidase, reactive and toxic aldehydes and H_2O_2 were shown to be responsible for the lethal effect of elevated spermine levels. Immunolocalization studies with antiserum against the polyamine oxidase revealed an abundance of the enzyme in the hypodermis and possibly in the cuticle of *O. volvulus*, a result which together with the demonstration of polyamine oxidase in the spent medium of maintained *O. volvulus*, point to an outstanding function of the polyamine oxidase at the interface of parasite and host.

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Presentation of EC supported joint research projects (1991-1996) continued
STD3
INCO-DC: 1st and 2nd Call

Areas of interest:

Contract number: ERBTS3*CT910038

IDENTIFICATION OF CANDIDATE PROTECTIVE MOLECULES OF *E. GRANULOSUS* AND DEVELOPMENT OF COMBINED *SALMONELLA* VACCINES

Period: May 1992 - October 1995

Co-ordinator: UNIVERSIDAD DE LA REPUBLICA, FAC. QUIMICA DE IMMUNOLOGIA, Montevideo, Uruguay (A. NIETO CADENAZZI)

Objectives

Long term aim is development of effective vaccines for hydatidosis and immediate aims are:

- The identification of vaccine candidate *E. granulosus* antigens (Ags) through analysis of immune evasion mechanisms as well as isolation of recombinant clones encoding the relevant Ags from cDNA libraries. Ags to be investigated will include *glutathione-S-transferase* (GST) and myosins. Immunogenicity and protection will be tested in animal models.

- The analysis of the structure of *E. granulosus* glycans and their influence, as well as that of the idiotype network, on the balance between susceptibility and protection to infection.

- The expression of recombinant antigens in live *Salmonella* vaccines to obtain maximal expression of the recombinant antigens in an integrated and maximally immunogenic form.

Activities

The Uruguayan partner (Montevideo) will:

* purify the *E. granulosus* metalloproteases;

* purify anti-complement molecules obtained from *E. granulosus*;

* identify putative protective antigens from protoscolex (PSC) surface antigens used to protect mice;

* assay the immunogenicity of those antigens; those exhibiting protective capacity will be cloned in the laboratory of the UK partner (Newcastle);

* identify and characterise immunogenic parasite glycans and analyze their role in immune evasion as well as that of anti-idiotypes;

* analyze the influence of *E. granulosus* on Th1/Th2 balance and susceptibility to infection.

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The UK partner (Newcastle) will be responsible for:

* the optimisation of expression of recombinant antigens in aro and htrA *Salmonella* vaccines. The antigens will be expressed both as the whole protein and as immunogenic peptide fused to LT-B;

* the testing of the construct in the mouse model for developing humoral and cell-mediated immunity.

The French partner (Paris) will participate in collaboration with Prof. Ehrlich's laboratory in the search of genes coding for proteins essential for parasite survival.

Expected outcome

E. granulosus protease clones will become available for use as immunogens and for sequence analysis. Complement activating glycans will be functionally characterised and the *in vivo* role of complement in susceptibility to infection will be tested. Candidate protective molecules and specific antibodies (Abs) will be prepared. Putative protective molecules will be cloned in *Salmonella* and tested as immunogens. Optimisation of expression of recombinant antigens in *Salmonella* vaccines will lead to the development of a putative *Salmonella*-hydrated vaccine, using the recombinant hydatid antigens which become available.

Results

Uruguayan partner

E. granulosus metalloproteases (EgMP) cleaving human IgG3 and IgG1 were purified. Abs recognizing the 75kDa EgMP band, were found in sera from infected mice and humans but not dogs. Rabbit anti-EgMP was used to screen a cDNA library. A clone was purified, but no sequence homology was found with any known protease. Complement activation at different steps of the cascade by different parasitic preparations was assayed Cyst fluid (CF) produced the maximum TCC levels. CF derived *N*-linked oligosaccharide which produce TCC by fluid phase complement activation were identified. In addition, *in vivo* complement depletion was shown to decrease susceptibility to infection in mice. At least four glucoproteins from the protoscolex (PSC) surface have been identified which are recognised by sera from protected mice and included in ISCOMs which were immunogenic intranassally. Parasite GST and a candidate protective *E. granulosus* clones, one homologous to tropomyosin (EgDf5) and the other to fatty acid binding proteins (EgDf1) were isolated in Prof. Ehrlich's laboratory in collaboration with Prof. Nieto's laboratory. The role of CD4+ and CD8+ T-cells in immunity as well as the IL profiles they produce during *in vitro* proliferation were also analyzed in the mouse in collaboration with Dr. Anders Örn (Karolinska Institute, Stockholm). A MAb (E492) was prepared recognizing Gala1-4Gal in PSC and used to isolate a fraction, containing complement-activating, mitogenic, and immunodominant T-independent glycan Ags. Surface PSC glycans obtained by EndoF treatment, were mitogenic *in vitro* and produced hypergammaglobulinemia *in vivo.* They were immunodominant in mouse, eliciting low avidity Abs and an unexpected IgM titer increase after booster. Cyst membranes (CM) glycans were also characterized and found

to contain both *N*-linked and *O*-linked oligosaccharides which are immunogenic in infected hosts. A MAb (2B5) recognizing the immunodominant Gal-gicNAc-Man motif in *N*-linked oligosaccharides from CM was prepared. Four major *N*-linked oligosaccharides were found containing GlcNAc, Man, Fuc, Gal and NeuAc. Their structures were further analyzed by FAB-MS in collaboration with Prof. Anne Dell (Imperial College, London). The role in immune regulation of anti-idiotypes mimicking CF Ags was analyzed.

UK partner

A student from Prof. Nieto's laboratory completed his PhD degree in Cambridge performing this research. Methods for the expression of recombinant antigens in *Salmonella* vaccines as fusion proteins to fragment C of tetanus toxin (TetC) were optimised. Preimmunisation with tetanus toxoid did not suppress the response to guest antigens presented as such fusions to TetC. He expressed glycoprotein D of herpes simplex virus in that system and obtained protection of mice from challenge with virus. As fatty acid binding proteins conferred protection from fascioliasis and schistosomiasis, EgDf1 was considered candidate for protection in *E. granulosus*. EgDf1 was cloned and expressed in *Salmonella* and an htrA vaccine expressing it was orally administered to be used for expression of EgDf1 as above and its protective potential in dogs will be tested in Uruguay.

French partner

Prof. Scazzocchio has participated in collaboration with Prof. Ehrlich's laboratory as described above.

Contract number: ERBTS3*CT910038

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Contract number: ERBTS3*CT910039

MOLECULAR APPROACH TO ECHINOCOCCUS DEVELOPMENT

Period: July 1, 1992 - September 30, 1995

Co-ordinator: UNIVERSIDAD DE LA REPUBLICA, FACULTAD DE CIENCIAS, Montevideo, Uruguay (R. EHRLICH)

Objectives

The main objectives of the project were the understanding of the molecular bases of:

- growth and development processes that take place during the *E. granulosus* life cycle and
- the mechanisms involved in the adaptation to its specific hosts. The leading concept of the proposal was that the knowledge of the regulatory processes controlling *E. granulosus* development, growth and host adaptation could made important contributions to hydatid disease control.

Furthermore, a complementary objective was proposed: it was focused on the optimisation of the expression of specific parasitic antigens in bacterial vectors, in particular in *Salmonellae*, attempting to contribute to the production of anti-parasitic live vaccines.

Activities

The study of *E. granulosus* growth and development have been undertaken focusing on the following aspects:

* Search for transcription factors able to be involved in the regulation of developmental events during the parasite life-cycle;
* Characterization of developmentally regulated genes in order to establish initial molecular markers to decipher regulatory mechanisms;
* Analysis of the parasitic-host adaptive processes through the study of the heat shock response;
* Study of cytoskeletal protein genes;
* Cloning and expression of antigen encoding genes;
* Analysis of the parasite's genome organization;
* As a complementary objective, the improvement of the expression of parasitic genes in attenuated *Salmonella* have been also undertaken.

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Expected outcome

Major contributions expected from this project included:

⇒ Contribution to deciphering the basis for the specific host-parasite adaptation and characterising key genetic switches during parasite development;

⇒ The improvement of the expression of Platyhelminth genes in *Salmonella*;

⇒ Training of Latin American scientist in molecular approaches to parasitic diseases.

Results

In a first step we have completed a great deal of descriptive work, mainly involving isolation, cloning and sequencing of several genes. During a second period, the main effort has been centred on expression studies (characterization of promoters, mapping of transcription start sites, improvement of different approaches to study complex formation between promoters and specific transcription factors, spatial and temporal analyses of gene expression).

Concerning molecular studies of development, growth and adaptation of *E. granulosus*, the following work has been carried out by our groups:

⇒ Characterization of transcription factors possibly involved in regulation of developmental events during the parasite life cycle: five homcobox- containing genes have been described: Eghbx1-5. The expression of two of these genes has been detected in protoscoteces (Eghbx1-2); in particular Ebhbx1-3 appeared to be expressed specifically in cells associated to calcareous corpuscles;

⇒ Isolation of two protoscolex differentiation markers: EgDf1, a gene coding for a protein related to the fatty acid binding proteins family (FABPs) and EgDf5, coding for a protein related to tropom-yosins. The EgDf1 protein could be involved in the binding and transport of lipids from host origin, a function of crucial importance for an organism like *E. granulosus*, unable to synthesize *de novo* most of its own lipids. Proteins related to the FABI's family were also described in *Schistosoma mansoni* and *Fasciola hepatica*; it was reported that both proteins are able to induce significant protection against experimental infection in animal models. The Egdf5 gene appeared to be expressed specifically in cells of the protoscolex suckers;

⇒ With the aim of studying the mechanisms of parasitic adaptation to the specific hosts, the heat shock response and its time course in *E. granulosus* has been characterized. Several stress proteins were identified by 2D-electrophoresis and a genomic clone containing the complete coding region of a Hsp70 protein, including the 5 regulatory domain was characterized;

⇒ Several genes coding for proteins involved in cytoskeleton organization were characterized; three different actins, the troposyosin-like protein mentioned above, and a gene coding for a putative actin-filament-fragmenting protein.

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⇒ Two genes coding for enzymes have been characterized a cytocolic malate dohydrogenase gene and a gene coding for a thioredoxin-like protein.

⇒ A gene coding for calcium-binding protein (EgCaBP) was isolated and its expression has been focalized at the level of calcareous corpuscles.

⇒ Six putative RNA polymerase II transcription regulatory domains have been identified in Porto Alegre and Montevideo labs; they include the proximal promoter domains of EgDf1, Eghbx,1, two actions, malate dehydrogenase and a hsp70 gene. Several targets for general and specific transcription factors were identified and some conserved sequences, that could constitute the binding site for specific protoscolex transcription factors, were detected.

⇒ In relation with *E. granulosus* genome organization, a middle repetitive sequence organized like a mobile element has been reported and the structure of a functional rDNA gene and its regulatory domain have been recently established.

Finally, with respect to the complementary objective of the proposal, the UK team developed a system which allows expression of recombinant antigens in live *Salmonellae* as either full length proteins or multiple tandem copies of immunogenic epitopes as C terminal fusions to the immunogenic fragment C of totanus toxin, under the control of the anaerobically inducible nirB promoter. Using this system, a trivalent experimental *S. typhimurium* vaccine has been constructed, which protects mise from typhoid, tetanus and schistosomiasis following a single oral dose of the vaccine. The EgDf1 gene from *E. granulosus* has been expressed using the system described above. Preliminary results indicate that the construct is immunogenic in mice.

An important number of publications have been produced, from which several correspond to joint articles. The number of young Latin American scientist trainee within the frame of the project was equally important: 5 PhD have been completed either in the L.A. or in the European laboratories and also in "sandwich" programmes including work carried out in both sides; finally 9M. Sc. degrees were completed in the L.A. laboratories. The collaboration between all the partners has proved very fruitful not only in promoting North-South and South-South transfer of expertise, but also in building up research capability in both DC labs. A multidisciplinary collaboration was established through the interaction with another project centred on immunobiological aspects of hydatidosis (TS3*CT910038). The Latin American partners were also involved in the recent launching of a Network for Research and Training in Parasitic Diseases at the Southern Cone of Latin America. Finally, a FAO Collaborating Centre on Epidemiology, Diagnosis and Control of Echinococcosis/Ilydatidosis has been created in Montevideo, including the partners of projects TS3*CT910038 and 0039.

Contract number: ERBTS3*CT910039

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Contract number ERBTS3*CT940270

HUMAN CYSTIC AND ALVEOLAR ECHINOCOCCOSIS IN NORTHWEST CHINA: COMMUNITY SCREENING, CASE FOLLOW-UP AND TRANSMISSION STUDIES

Period: January 1, 1994 - December 31, 1996

Co-ordinator: UNIVERSITY OF SALFORD, United Kingdom (P. CRAIG)

Objectives

- Screen populations at risk and measure exposure and disease in two selected rural communities in China where either cystic or alveolar echinococcosis is predominantly endemic, using a combination of immunoserology and portable ultrasound scanning.
- Undertake immunoserological and ultrasound follow-up on hydatid patients and asymptomatics.
- Quantify transmission potential of dog definitive hosts in the study communities and elucidate the major life-cycle of *E. multilocularis* in south Gansu.

Activities

Human echinococcosis (hydatid disease) is a major problem in northwest China especially in Xinjiang and Gansu Provinces. Both *Echinococcus granulosus* and *E. multilocularis* are endemic in these provinces and responsible for some of the world's highest human prevalence rates. One community was investigated, in each province, where transmission of either cystic (Tacheng, Xinjiang) or alveolar echinococcosis (Zhang, Gansu) predominates. Between 1500-2000 people in each community were screened using combined ultrasound scanning and serology. Image positive and/or seroreactors (against cyst antigens) were followed up serologically (primarily including IgG1 and IgG4 antibodies) before treatment (medico-surgico) during treatment and up to 12 months post-treatment. A natural history of human alveolar echinococcosis has begun to be described based on ultrasound and serological data. Risk factors were identified using a questionnaire. The role of the domestic dog in transmission of cystic echinococcosis was assessed through immunodiagnostic tests (coproantigens) in conjunction with parasitological prevalence and rate of re-infection studies (after praziquantel treatment). For each community the primary transmission cycle(s) and resultant risk of human exposure and infection, were investigated. Human and animal data (parasitologic and immunologic) were analyzed to enable construction of transmission pattern(s), including rodent parasitological and ecological studies for *E. multilocularis*.

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The long term aims include recommendation of appropriate hydatid disease screening, surveillance and treatment follow-up strategies for Chinese rural communities, and to consider optimal approaches for monitoring regional control programme or disease transmission within defined regions.

Results

This joint research project has already achieved most of the original expected outcomes. The most important to quantify the human infection and disease rates of alveolar echinococcosis (AE) in south Gansu and cystic echinococcosis (CE) in north west Xinjiang; also to establish optimal community based screening and the successful process of training Chinese scientists in UK and France.

In south Gansu province (Zhang County) people from 16 villages were screened for AE by hepatic ultrasound. In addition 3 more villages screened prior to 1994, and 8 villages (screened in 1996) from the Zhang - Min County border were included. Thus - 2000 people from 27 villages in the region are included in the Gansu study. The total human AE prevalence rate was 5% with a mean village prevalence rate of 4.5% and a range of 0% to 15.8%. These AE rates were extraordinarily high compared to other important endemic areas such as Alaska, Siberia and Western Europe.

Two major observations derived from the study. The resultant large numbers of AE patients (>100) presenting with early or advanced lesions provided a unique data set to consider the evolution of AE disease. Disease probably progresses from small hemangioma - like lesions through to hyperechoic lesions and advanced disease with large hyperechoic areas with central cavitation. The study also indicated through specific antibody assays that natural resistance to AE probably occurs which exhibits as punctiform, linear or circular calcifications. Interestingly a predominant proportion of early AE cases were IgG1 rather than IgG4 antibody positive, while advanced cases exhibited both IgG1 and IgG4 sero reactivity. Full immunoserologic profiles will be obtained from the patient panel.

The other important observation in Gansu was that of the significant correlation between human AE village prevalence rates and the village landscape profile. Land-use indices showed that villages close to areas with high ratio of scrub or rough pasture had significantly higher human AE rates, while villages surrounded by larger areas of ploughed field exhibited much lower disease rates. The densities of *Microtus oeconomus*, a potential rodent intermediate host of *E. multilocularis*, were significantly correlated with the latter village landscape profiles.

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The distemper induced dog population crash in the region between 1991 and 1994 prevented a study of *E. multilocularis* in that host.

However, our hypothesis is that human AE infection rates reflect transmission 10-15 years in the past, when the dog population density was higher. Furthermore, known cyclical changes in Microtus spp. which coincide with increased canid populations (dogs and foxes) lead to local increases in transmission within both wild (fox-rodent) and semi-domestic (dog-rodent) cycles. Over time (decades) gradual deforestation in the upland areas probably initiates increased cyclicity of *M. oeconomus*, which in close association with marginal villages increases local transmission of *E. multilocularis*. Eventually with increased area under plough microtine densities will decrease together with predicted AE rates.

Parallel collaborative studies in Tacheng prefecture, Northwest Xinjiang revealed a very different pattern of echinococcosis amongst community of predominant mongolian pastoralists residing close to the Kazakhstan border, where cystic echinococcosis is the dominant endemic parasitic disease. Screening of 1823 persons by ultrasound, serology and questionnaire revealed a CE rate (due to *E. granulosus*) of 3.9% (71/1823).

36% of the population owned a dog, which increased to 100% of the families who moved to the summer mountain pasture at Kokehada. Preliminary analysis showed that 77% of CE cases were seropositive against antigen B and 28.5% of persons who exhibited isolated calcifications. Antibody IgG sub-class analysis is currently being undertaken. Domestic dog population in both winter and summer pastures is being screened by the Echinococcus coproantigen ELISA test developed by the research team. In contrast to south Gansu, the pastoral area of Kokehada was dominated by the ground squirrel *Citellus erythrogenys* whose status in transmission of *E. multilocularis* requires confirmation. Also no advanced AE cases were detected during the screening but 2 putative cases with small hepatic lesions were identified for follow-up.

The resultant cooperation that has developed between Salford University and University of Franche-Comté (with the associated partner - University of Bourgogne) has been very successful. This new European based cooperation has also resulted in development of collaborative research on the transmission of *E. multilocularis* in Eastern France. Furthermore, prior to this STD3 project the French research team (unlike the UK group) had no collaborative projects in China. Now as a result a separate Franco-sino ecology project has developed with Xinjiang and Lanzhou Medical Colleges. Also, within China, the move toward inter institute and inter-provincial cooperation on echinococcosis has been set in motion.

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A northwest provinces research meeting (Xinjiang, Gansu, Qinghai, Ningxia and Sichuan) on echinococcosis was very successfully organized in Lanzhou through the UK and France initiatives. As well as direct research cooperation and collaboration, training of 6 Chinese scientists (for periods 3m-12m) in areas of immunodiagnosis, ecology and clinical aspects has been successfully undertaken so far. Therefore the basis for not only continued research collaboration but also importantly self-sustained research programmes with Lanzyhou and Xinjiang Medical Colleges are now firmly placed.

Expected outcomes that were achieved wholly or in part

⇒ Quantification of the human infection and disease rates of alveolar and cystic echinococcosis in selected communities.

⇒ Establishment of appropriate community based patient follow-up procedures.

⇒ Identification of the probable main transmission cycles and risk factors for human echinococcosis in Gansu and Xinjiang.

⇒ Establishment of optimal community based screening strategies.

⇒ Training of Chinese scientists especially in immunodiagnosis and animal host parasitology/ecology, data handling skills.

⇒ Cooperation between UK and French Institutes and between Europe and China.

⇒ Recommendation at provincial, regional and national level for research and control of cystic and alveolar echinococcosis.

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Contract number ERBTS3*CT940277

CONTROL OF *TAENIA SOLIUM* CYSTICERCOSIS THROUGH SPECIFIC DIAGNOSIS, SYSTEMATIC EPIDEMIOLOGY AND DEVELOPMENT OF A RECOMBINANT VACCINE

Period: August 1, 1993 till July 31, 1996

Co-ordinator: UNIVERSITY OF EDINBURGH, CENTRE FOR TROPICAL VETERINARY MEDICINE, Edinburgh, Scotland (L.J.S. HARRISON)

General and long term objectives:

Taenia solium cysticercosis is responsible for serious public health problems, in addition to creating financial losses to pig producers in countries where the parasite is endemic. While control of the parasite can be achieved to some extent through improvements in public health, sanitation and pig management/husbandry practices, the development of reliable and sensitive diagnostic procedures would greatly assist control through facilitating the execution of reliable epidemiological surveys. Such surveys not only form the basis for pinpointing and evaluating control measures, but are also essential for the design of environmentally appropriate control strategies, including the introduction of a recombinant vaccine. The proposal therefore aims 1) to improve diagnosis of human and porcine cysticercosis 2) to conduct epidemiological surveys as a prelude to selecting appropriate study areas for assessing control via drug treatment and 3) select potentially protective antigens, for use in the design of a recombinant vaccine in a second phase of this project proposal. Of particular importance will be the detection of neurocysticercosis in man.

Specific objectives to be achieved by this proposal:

- To transfer established diagnostic procedures from Europe to Mexico, via a training programme.
- To conduct epidemiological surveys for porcine and human cysticercosis: the former in order to identify areas for control (e.g. by drugs such as praziquantel) and the latter as a guide to appropriate medical treatment.
- To clone, sequence and express metacestode excretory/secretory proteins of diagnostic potential.

Activities

* To transfer established procedures from Europe to Mexico via a training programme. A Mexican student will be trained in the use of a monoclonal antibody based antigen detection ELISA assay developed in UK. The assay will then be transported to Mexico where it will be standardised for use in the detection of human and porcine cysticercosis followed by epidemiological studies. A follow up visit by a member of the European component is then to be carried out to Mexico once the student has returned to the laboratory.

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* Existing DNA probes for the differentiation of *T. solium* and *T. saginata* will be sequenced and developed into a PCR diagnostic test for use in the field.

* To conduct epidemiological studies for porcine and human *T. solium* infection. The immediate objectives of this study are to carry out a survey in pigs reared under different management systems, comparing the results obtained with the ELISA assay with the presently used meat inspection procedures and detailed tongue examinations in pigs. At the same time studies will be conducted to determine the efficiency of the assay and to determine sero-prevalence in hospital patients.

* To clone, sequence and express potentially protective oncospheral genes. Due to the known extensive cross reactions between *T. solium* and *T. saginata*, and to the hazards of working with *T. solium* oncospheres, the identified potentially protective oncospheral antigens will be cloned from (λ-Zap (Stratagene)) cDNA libraries of *T. saginata* oncospheres.

* To clone sequence and express excretory/secretory proteins of diagnostic potential. The first activity, which was already achieved, was to identified the protein antigens which were to be cloned; the second to prepare or collect suitable serum samples for use in the primary and secondary screening of the cDNA library; the third activity is to prepare a cDNA library from *T. saginata* metacestodes. Finally, any identified clones from this library and the library prepared from *T. saginata* oncospheres will be recloned into a suitable vector for more efficient expression. Clones first identified using the *T. saginata* system will be subject to secondary screening in order to identify that sub-set is also reactive with *T. solium.*

Results

Training

The newly prepared HP10 monoclonal antibody reagents were titrated and standardise prior to shipment. The Mexican student trained in the conduct of the ELISA assay and lyophilised reagents were transported back to Mexico, lyophilised for use in the screening work. DNA probes were grown up and sent to Spain for sequencing and further analysis.

Epidemiological studies for porcine and human T. solium infection

A collection of sera from pigs and human either *T. solium* infected or non-infected were collected in order to evaluate the sensitivity and specificity of the diagnostic ELISA assay.

A group of 293 sera from non-infected (49) and experimentally infected pigs (244) were obtained (from pigs experimentally infected and maintained in the Veterinary School in UNAM).

Pigs lightly or heavily infected were bled at different times during the infection and, after the number of cysticerci were determined in a representative sample of each pig (to obtain sera from rustically bred pigs), we examined different slaughter houses near Mexico city, and identified one in Zacatepec, Morelos, which introduces a considerable amount of rustically bred pigs from the states of Puebla and Morelos (Mexico). This is of a special interest considering that this is the population exposed to the higher risk to the infection. With the support of the authorities of this abattoir, we collected 200 ml of sera for each pig and also their tongues.

Tongues were maintained in formol saline and the parasite number determined by slicing the tongue to count all the cysts present. Cysticerci collected were conserved for confirmation by an immunopathologist. In addition, a panel of 32 sera from a slaughter house in which only pigs from technified farms were included were used to test serological assays, sera from rustically bred pigs from Tianguizolco, Guerrero. A panel of 43 sera from Tianquizolco were collected. These 43 pigs were randomly selected and completely necropsied to determine the presence of cysticerci or other disease. A collection of 112 CSF were obtained from the Institution Nacional de Neurologia y Neurocirugia, Mexico, D.F. For each patient the diagnosis was confirmed based on the clinical examination, nuclear magnetic resonance and tomography. The pathology and type of infection was also recorded. Finally we prepared a collection of human sera from a neurological institution (Instituto Nacional de Neurologia y Neurocirugia, D.F.). For this, we collected 392 sera from patients that consulted the institution for the first time. For each the sex, age, clinical diagnosis, AIDS, NMR, TC, and other pathology and infections were recorded.

All the results obtained in the evaluation of the assays based on the detection of antigen HP10 and antibodies against vesicular fluid antigens indicate that both assays are appropriate to cysticercosis diagnosis in pigs maintained in technified conditions. However, both assays showed a lower specificity and sensitivity for the detection of cysticercosis in rustically bred pigs and infected humans.

Several clones have been identified and sequenced from the *T. saginata* oncospheral library including the gene encoding the principal 18kDa secreted antigen of activated oncospheres of *T. saginata*. The sequence and immunogenicity of *T. saginata* ferritin has been established. Various expression systems have been examined with a view to selecting the most promising for the larger scale expression of *T. saginata* proteins. The selected systems are now functional in the IAH Pirbright laboratory, where preliminary experiments have been initiated.

For the cloning, sequencing and expressing of excretory/secretory proteins of diagnostic potential, groups of calves were infected with *T. saginata* metacestodes in order to produce 4 week old metacestodes. These were extracted and used in the preparation of a λZAP (Strategene) cDNA libraries on three separate occasions. Libraries are constructed according to routine procedures. Metacestodes of this age have been shown to produce diagnostically important excretory proteins. The intention is to use these protein antigens as the trapping layer in ELISA assays designed to detect anti-parasite antibody in the serum of infected cattle.

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The initial extraction and preparation of the RNA was conducted at CTVM while the cDNA preparation and titration of the resultant libraries was conducted at IAH Pribright. Once the libraries were prepared they were screened with sera in Madrid.

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Contract number ERBTS3*CT930219

FIELD EVALUATION AND FURTHER CHARACTERIZATION OF AN INVASIVE-SPECIFIC MONOCLONAL ANTIBODY AGAINST *ENTAMOEBA HISTOLYTICA*

Period: January 1, 1994 - December 31, 1996

Co-ordinator: LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE, DEPT. OF MEDICAL PARASITOLOGY, London, United Kingdom (D. WARHURST)

Objectives

Application and development of techniques for distinction of *Entamoeba histolytica* and *E. dispar*, with a view to clarifying the epidemiology of amoebiasis in different areas of the world, and focussing the treatment effort. Training and transfer of technology, to enhance research potential of scientists from countries with endemic diseases.

Activities

Training visits, collaborations and joint publications.

Results

A colorimetric PCR technique (Solution Hybridisation Enzyme Linked Assay (SHELA) has been developed for differentiation of faeces containing *E. histolytica* and *E. dispar*. A comparison of the zymodeme of culture of Bangladesh isolates with SHELA results on culture in Bangladesh was agreement in detection of *E. histolytica* in 23/23 cases (13 zymodeme 2, and 10 zymodeme 14). However, three cultures identified as zymodeme 1 were tested and 2 reacted in the SHELA as *E. histolytica* and the other one as *E. dispar*. It is clear more testing of non-pathogenic material from Bangladesh is needed to determine whether this degree of non-agreement is significant, since it has not been seen in material from other areas. It is encouraging to note that zymodeme 14 reacts as well in PCR-SHELA as zymodeme 2.

The parallel examination of the original faecal specimens using the *Entamoeba* and *E. histolytica*-specific ELISA technique of Petri and colleagues gives some contradiction with zymodeme and with the PCR-SHELA. The common occurrence of apparently mixed infections is notable. However, given that the original faeces is being examined and culture is inevitably selective, this is not so much of a problem as the rather low sensitivity of the immunological technique for detection of *E. histolytica* itself. To investigate this further 40 more faecal specimens from Bangladesh are being tested in the UK laboratory and will be compared with the results of culture and of the Petri ELISA.

Improvements to the protocol and kit for the PCR-SHELA have been developed. Using a modified system named Demi-Nested PCR-SHELA. This has been successful for both amoebiasis and malaria context. The advantage of the technique is that the labelled internal probe, which normally needs to be added to the PCR product after the reaction, is incorporated in the sealed tube, and acts as a primer of a PCR nest, and as the detector for the 1st and 2nd products of the PCR.

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All that needs to be done at the end of the single PER run is to dilute the product in buffer and apply it to the microtiter plates for the avidin capture and digoxigenin enzymatic detection procedure. This substantially reduces the time taken for the technique.

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Contract number: ERBTS3*CT930227

IMMUNOLOGICAL CORRELATES OF RESISTANCE AND SUSCEPTIBILITY TO INFECTIONS WITH GASTRO-INTESTINAL NEMATODES IN NORTH EAST BRAZIL

Period: January 1, 1994 - December 31, 1996

Co-ordinator: UNIVERSITY OF NOTTINGHAM, DEPT. OF LIFE SCIENCES, Nottingham, United Kingdom (D. WAKELIN)

Objectives

The overall objective of the proposal is to make a detailed study of the immune responses to infection with gastro-intestinal nematodes and to identify causal correlates of resistance and susceptibility to these important parasites. The specific objectives are:

- Determine the prevalence and intensity of gastro-intestinal nematode infections across an appropriate age range in populations living in communities where these parasites are endemic.
- Follow the patterns of reacquisition in these populations after effective chemotherapy.
- Identify and characterize individuals showing resistance or susceptibility to infection and re-infection.
- Analyze the antibody, cellular responses to each infection, correlate these with parameters of resistance and susceptibility, and identify target antigens of the parasites concerned.

Activities

Infections with the major gastro-intestinal nematodes of man are endemic in the Recife area of N.E. Brazil, a pilot study showing high prevalence in poor urban and rural communities, and the excellent laboratory facilities at CPq AM, Recife offer a unique opportunity to examine the parasitology and immunology of these infections. Collaboration with the laboratories in Nottingham and Marseille provides an unrivalled combination of expertise in the immunoparasitology of helminth, specifically intestinal nematode, infections. The proposal is to carry out a detailed study of immune (serological, cellular and cytokine) responses in individuals of known infection/reinfection status. The data obtained will make it possible to correlate resistance or susceptibility to infection with the capacity to mount particular responses, and throw light on those mechanisms which regulate the development and expression of host protective immunity. In this context definition of T helper subset responses, and of target parasite antigens are seen as key priorities.

Expected outcome

The project has provided the first detailed survey of intestinal nematode infections in N.E. Brazil. Extensive clinical data on the surveyed communities have been collected and are being analyzed. The populations have been extensively surveyed, blood and stool samples have been taken on several occasions. It is clear that the parasites *Ascaris*, Hookworm and *Trichuris* have high prevalence, particularly in children.

Contract number: ERBTS3*CT930227

Worm burdens: particularly in the case of *Ascaris*, can reach very high levels and are therefore likely to cause a number of clinical problems. The populations have been treated with anthelmintic and the patterns of reinfection followed. Lymphocytes and sera from individuals shown at each survey to be repeatedly heavily of lightly infected are being analyzed for antibody and cytokine responses in order to look for immunological correlates of resistance and susceptibility. The data collected have already contributed to increased public health awareness of the importance of intestinal nematode infections in the Recife area. The research capabilities of the Brazilian partner have been considerably strengthened. Surveys carried out in poor urban and rural areas in the Recife area showed a high prevalence of gastrointestinal (GI) nematode infections.

In the urban area *Ascaris lumbricoides* and *Trichuris trichiura* were the commonest species (57 and 62%), whereas in the rural area hookworm (*Necator*) was commonest (79%). Infections were present in all age groups, but were most prevalent and most intense in children below 16 years. Only Ascaris infections occurred at very high intensity, faecal egg counts of more than 20,000 eggs per gram being recorded. The study groups have all received chemotherapy, and this proved largely successful in eliminating infection, but with time since treatment levels of infection are again rising. Sera taken before the first chemotherapy have to date been analysed for total and parasite specific IgE responses, as this isotype is considered to give the greatest degree of parasite specificity. High levels of total and parasite specific IgE have been recorded in individual infected with *Ascaris* and with *Trichuris*. Although the first result was unexpected, high anti-*Trichuris* IgE responses were not. Assays for IgG isotypes are now in progress, and it is hoped soon to have data on cytokine responses from individuals who have consistently shown either high or low worm burdens. All of these data will then be analysed for association with level of, and predisposition to, infection.

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Contract number ERBTS3*CT940294

INTEGRATED MULTIDISCIPLINARY STUDY OF HUMAN FASCIOLIASIS IN THE BOLIVIAN NORTHERN ALTIPLANO

Period: October 1, 1994 - September 31, 1997

Co-ordinator: UNIVERSIDAD DE VALENCIA, DEPT. PARASITOLOGIA, FAC. FARMACIA, Valencia, Spain (S. MAS COMA)

Objectives

Characterization of human fascioliasis in the Bolivian Northern Altiplano (in humans endemic with very high prevalences, very high altitude) in preparation for control measures. Important related objectives are interaction and transfer of technology between European partners and the INLASA Institute of the Bolivian Health Ministry, as well as transfer of personnel from the Bolivian Health Ministry, to the European Community laboratories and *vice versa.*

Activities

Research includes field and laboratory work for:

* Biological characterization of parasite and snail vectors.
* Assessment of the present situation of human fascioliasis and vector geography.
* Development of a new immunodiagnostic assay for the detection of human and animal fascioliasis.
* Application of the new immunodiagnostic assay in the Bolivian Altiplano endemic area.

Moreover in the first two years, several Bolivian professionals have participated in laboratory research activities of the project in European centres.

Results

Concerning the first two objectives the main results obtained in the first two years are:

⇒ The endemic zone appears to be isolated between the Lake Titicaca and La Paz (about 250.000 people living in the zone: more than 2 million people in the neighbourhood; boundaries marked by geographic, climatic and soil-water chemical characteristics; large livestock population at risk), and the transmission foci are stable (maintained both during the year and along pluriannual periods) and patchily distributed.

⇒ The parasite has developed several strategies to adapt to the altitude conditions which favour transmission (very long cercarial shedding period, assuring a more prolongated infection of the environment with free-living metacercariae; very high cercarial production, very great survival capacity of the intramolluscan larval stages, production of floating metacercariae. Therefore drinking fresh-water may serve as an additional mode of infection, very large infection capacity of metacercariae and adults, normal development in the pig and very high transmission rates of pig isolates at molluscan level, high prevalence in cattle and sheep, also numerous additional potential domestic and wild definitive hosts are present)

⇒ The transmitting snails have proved to belong to only one species, the European species *Lymnaea truncatula* imported by European colonizers, appears to have developed several strategies to adapt to the altitude conditions which favour transmission (markedly precocious sexual activity; high reproduction capacity, very rapid development chronology, long survival capacity, large adaptation capacity to extreme conditions, postparasitation viability linked to permanent water collections permitting transmission during the whole year, enzymatically identical populations with total absence of polymorphism).

⇒ Man has proved to be a very effective and viable definitive host (human isolates show very high transmission rates at molluscan level), with very high prevalences (up to 90.0% by serology and 66.7% by coprology in the total population and up to 38.2% by coprology among school children), very high individual infection levels (amounts of more than 1000 eggs/g faeces are numerous in children; even up to 5064 eggs/g in extreme cases), and with up to 12 different protozoan species and 5 helminth species concomitantly infecting fasciliasis-infected children, including well know pathogens, as *Entamoeba histolytica, Cryptosporidium sp., Giardia intestinalis, Ascaris lunbricoides or Trichuris trichiura.*

⇒ Andean inhabitants show several customs related to transmission (eating of aquatic or semiaquatic vegetables by adults, swallowing or chewing of aquatic plant stems and roots by children, defaecating in the external environment, tradition of each family sustaining its own livestock, including cattle, sheep, pigs and horses or donkeys, a custom of great social settledness among Aymara Indians, life in dispersed communities).

Concerning the last two objectives main results obtained in the first two years are:

⇒ Two cysteine proteinases secreted by adult *Fasciola hepatica* (from bile ducts of infected bovine livers) were isolated, characterized and prepared by immunologically screening; they were designated cathepsin L1 (27 kDa) and cathepsin L2 (29.5 kDa).

⇒ Immunoblotting experiments demonstrated that prepared antisera specifically detect cathepsin L. Proteinases in extracts of adult *F. hepatica*.

⇒ Adult fluke cDNA library was prepared and cDNAs encoding the two cysteine proteinases cathepsin L1 and cathepsin L2 isolated.

⇒ When working with sera samples obtained from 96 native Aymara living in the Bolivian locality of Calasaya, cathepsin L1 (Cathepsin L1) proved to be more reliable for the detection of infected individuals than liver fluke extracts and was as good as or better than ES product, the predominant antibody isotypes elicited by liver fluke-infected humans are IgG1 and IgG4.

⇒ By exploiting this fact an ELISA was developed which detects anti-IgG4 reactions to cathepsin L1, this assay shows a 99% reliability in detecting infected humans.

⇒ *F. hepatica* cathepsin L1 has been successfully expressed and has functional and physicochemical properties in common with the native proteinase produced by adult liver flukes; immunoblotting experiments showed that the yeast-expressed molecule is also reactive with antibodies prepared against the native enzyme.

Contract number ERBTS3*CT940294

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Annexes:

ANNEX 1:

PARTNERSHIP BETWEEN EUROPE AND DEVELOPING COUNTRIES IN HEALTH RESEARCH[1]

The European Commission has been supporting joint research activities relevant to development, including health related research, since 1983, and has recently issued the second call for scientific proposals within the programme of International Cooperation with Developing Countries (INCO-DC) which has become a broader programme than its predecessor. Health research remains a priority area, together with research on agriculture and the sustainable use of natural resources. The Commission's International Scientific Cooperation Programme is an integral part of the European Union's Fourth Framework Programme on Research. It serves Europe's research policy but it does even more. It is also an instrument to support other European policies, such as development cooperation, external relations or economic cooperation with third countries.

A guiding principle of the European Union is a desire to contribute to worldwide sustainable development. A desire which stems from a sense of co-responsibility for the problems faced by third countries. This responsibility must be shared by every citizen but a special role, undervalued in the past, must be reserved for the scientists who have chosen to address problems of developing countries.

The most important commodity for sustainable development is knowledge and knowledge will be the number one production factor in the 21st century. Its importance will far outweigh that of capital and labour in the present century, and if one wishes to safeguard the future and to increase knowledge, more needs to be invested in research now. Research is increasingly perceived as a basis for welfare within the EU. Research must therefore be just as important for the welfare of third countries. This assumption has received too little attention. Knowledge is an invaluable commodity and to acquire knowledge one has to help develop a culture of questioning.

Responsibility and Mandates

The development of a culture of science and research is the responsibility of everyone. Scientists within the individual member states of the European Union are already involved at personal, institutional, regional, or national level in a variety of programmes with a variety of goals related to Health in tropical areas or developing countries. A wealth of research activities are, or can also be, performed or developed on a bilateral basis by each of the European Member States. The Commission strongly encourages all these efforts.

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[1] Reprinted with permission from: *Tropical Medicine and International Health* 1996 **5** 553-557.

Moreover, the Commission has a mandate to keep informed of any such initiatives and whenever possible to be in synergy with them, but the Commission's strength lies in its ability to harness expertise at a supra-national level within Europe, in the context of its relations with third countries. Adding a European dimension, complementing the existing bilateral regional and national interactions.

The European Commission will not replace any of the inputs from others. The more support scientists in Europe or Developing Countries receive or acquire from national or other sources, the more European programmes will be meaningful and give room for substantial cost efficient added value. The Commission functions on the basis of subsidiarity, a concept which has been a matter of debate in the EU. At the European level, the Commission does what can be done only at this level, and most importantly, done better at this level. The Commission builds on the valuable contributions to health research of the individual Member States of the European Union. Many agencies like the Institutions of the United Nations, such as WHO, have an alternative agenda focused on different roles and responsibilities, although often in support of common goals.

It is critical that each has their own clearly defined goals and modes of operation. It is also important that all operations are transparent so that what is being done, and where, can be precisely established, thus avoiding wasted efforts and unnecessary duplication. What it boils down to, is the need to establish equitable partnerships which will facilitate the sharing of knowledge. Clearly with the financial and human resources which can be harnessed from within the European Union the opportunity can be provided for partnerships to be established within Europe and with third countries. These resources are not an objective or a solution in themselves. They simply provide the appropriate environment and the means to progress.

For too long it was considered that ideas and practices in health research were "for" Developing Countries, and that they could be exported from Europe to Developing Countries for direct application. While that attitude was not exceptional in times gone by, it is now certainly no longer acceptable. The European Union encourages health research "with" Developing Countries.

In establishing partnerships in health research with Developing Countries, the criteria considered to be most important can be divided in three parts. The first is the scientific aspect. It is essential that the process of science is of a high quality, indeed of international status, and respected on its own merits and rules. There is only one science, whether it is classified as basic, fundamental, strategic, operational or action research. What matters is that science flourishes in a variety of socio-economic and cultural environments.

The second, but equally important consideration is the mechanism to implement the EU policy of scientific cooperation in health research. It would be naive to consider that any scientific interaction can take place in a vacuum, insulated from other aspects of life. There must be consideration of the societal aspects of the science. In other words there must be an expectation that the scientific work will, at one time, bring tangible benefits for society as a whole. Therefore, the work supported by the Commission is aimed at addressing the major health problems faced by developing countries. New practices and technologies have to take account of the context in which they may be applied and the health benefits of their application have to be clearly established. Public health concepts are a common platform for all health research.

The third important point to consider is that health research partnerships cannot be established unless there are partners to establish them with. Partners within Europe are easier to find, but research capacity in Developing Countries is relatively scarce and cannot be created overnight. Although the inputs from national or international research programmes can help, they are not the solution in themselves.

What is needed is a prolonged intensive investment and other support to ensure capacity and capability strengthening. This will only be achieved through the political will and the economic commitment of the countries themselves together with support from other national and international sources. Fortunately the need for this support has been recognised by the European Union through its economic and development cooperation policy which is mainly the responsibility of DGIB and DGVIII within the Commission.

Science should not suffer from any unproductive rivalry, as was the case in the early years of this century, when scientists argued over whether resistance to disease was dependent upon cells or serum. Scientists allied themselves on one side or the other on the basis of their nationality. In the initial stages of the European Union's Research Programmes even the prospect of uniting two European research institutions or laboratories from different member states seemed daunting to less receptive scientists, and brought some opposition. Scientists assumed, falsely, that their efficiency or competitiveness would be diminished by having to participate in what was considered by some to be a cumbersome interaction. But the imposition of this requirement of joint research for eligibility for European Union support has stood the test of time and is helping to change the paradigm for international cooperation to one of equitable partnerships.

The benefits this brings are many. Now, there is an unprecedented level of European cooperation with many examples of the EU programme providing the initial contacts between labs which have blossomed and been extended to address many problems outside the current programme. Competition between laboratories remains a driving force for advances, but this has ensured that scientists tended to become better specialists in their own particular area. There is now an appreciation that the complexity of the problems and challenges being faced, can be solved only through cooperation among scientists with expertise in complementary disciplines.

If establishing links between scientists in Europe needed some persuasion, European scientists found establishing links with developing countries even more difficult to accept. The EC is not interested in providing strictly pre-conceived "European" solutions to problems of development. There is an acute awareness that the complex problems of development cannot be contained within national or regional boundaries, they affect all societies. The aim is to find a common path to achieving improvements in development, bringing together scientists from North and South who will address the problems as an integrated unit, each bringing their own expertise and experience to bear on the problem at hand. If this is done in the right way, the goal of learning to learn will be achieved. A culture of learning, in which scientific methodology becomes an intrinsic part of society will be established. Hypotheses will be tested and development programmes modified in the light of the results, things will not be left to chance. The interactions of the scientists in the Commission's programme leads to the establishment of a culture of learning across the globe.

The Commission continues to encourage interactions among scientists. Links between European laboratories are stronger now than they have ever been. The same can be said for links between European scientists and their colleagues in Developing countries.

But there is an additional benefit from the requirements of the programme, South-South cooperation, and the sharing of knowledge among the third countries anxious to involve their regional neighbours in their quest for knowledge of common problems which are best addressed in partnership. Of course this is not a unique achievement but it is clear that the Programme has done much to facilitate additional steps in this direction.

ANNEX 2:

THE EUROPEAN UNION (EU) FOURTH FRAMEWORK PROGRAMME FOR RESEARCH AND TECHNOLOGICAL DEVELOPMENT (RTD)

Collaborative research with developing countries is carried out against the background of the Commission's Fourth Framework Programme for Research (FP4). The basic aim of FP4 is: To support inter-Member State scientific collaboration, networking and concertation on issues of common concern. To reach this goal FP4 supports multi-centre research projects, concerted actions and accompanying measures which help to improve quality of life and increase European competitiveness, in a global context. More than this, FP4 serves to support other EU policies, such as economic and development cooperation.

The total budget of FP4 (1994-1998) is 12.3[1] billion ECU and is divided over four main activities:

I. Research, Technological Development and Demonstration within the EU Member States

(10686 Million ECU (MECU)),

II. Cooperation with Third Countries and International Organisations (540 MECU),

III. Dissemination and optimization of results (330 MECU) and

IV. Training and Mobility of researchers within the EU (740 MECU).

Activity II, Cooperation with Third Countries and International Organisations has a pivotal role, linking EU policies in science, economic cooperation and development through coherent collaborative research activities with third countries.

Health-related research can take place in activity I and in activity II programmes. The BIOMED and the BIOTECH programmes of Activity I have a budget of 552 MECU and 336 MECU respectively. Many aspects of health research which are trans-disease, can be covered by these programmes and might also be of great importance for collaboration with Developing Countries for example, malaria vaccine development. Within the activity II INCO programme (540 MECU) there is also a health component. In the specific Programme for developing countries a total budget of 63 MECU will be available for health between 1994-1998 and in the component geared towards Eastern and central Europe, health-related research is also covered (INCO-Copernicus).

[1] In 1996, budgets of specific programmes are to be increased to the new total of 13.1MECU.

The Work Programme

Although they may differ in detail the Commission's research programmes in FP4 have the following modes of implementation:

I Multilateral joint research projects,

II Concerted Actions, covering the actual costs of concertation, such as the search for partners, meetings, common publications

III Accompanying Measures, such as contract holders meetings, networks, studies, targeted research training and mobility and the dissemination of results and

IV Concertation through consultation with the Member states and in the case of the INCO-DC programme consultation with developing countries.

These are set out in the detailed Work Programme together with the main objectives of the programme. For INCO-DC these are, to promote the role of relevant high quality RTD in development in economic cooperation, to encourage scientific collaboration between Europe and DCs, between DCs and within Europe, to help reinforce and maintain RTD capacities, including human capital, in DCs, to contribute to maintaining a competence in Europe in scientific sectors of mutual interest and in those pertinent to problems of DCs, to capitalise on the experience gained during the implementation of previous Commission S&T co-operation activities and to take into consideration the political obligations of the Union and the recommendations of international fora such as the Rio conference concerning research in DCs.

The Work Programme is implemented through Calls for Proposals which are updated from year to year. Details of research themes which are to be supported are provided in these Calls for Proposals.

The Call for Proposals

The Call for Proposals (Anon 1996) contains detailed information on criteria which have to be fulfilled by applicants, for example partnerships, and also on the specific topic areas for which research proposals are invited. The Call for Proposals is updated for each call and permits the Commission to direct the research programme on the basis of the consultation processes with the Member States, developing countries and on the Commission's existing research portfolio. In general the Call for Proposals is issued six months in advance of the deadline for submission of proposals. On arrival at the Commission, proposals undergo a stringent review procedure.

The Evaluation Process

In addition to scientific quality each proposal is evaluated on a variety of aspects, which include:

Is the health problem relevant to developing countries?
Is the problem of national, regional or global relevance?
Is the problem of known, documented magnitude?
Is the problem vulnerable (are there opportunities for cost-effective impact)?
Is there political will to overcome the problem?
Will the research be induce changes in approach or political awareness?
Does the research build on existing and matching capacities?
Does the research partnership have a comparative advantage (including DC)
Is there a demonstration value (spin-off in financial or scientific terms)?
Is there a likelihood of leverage for complementary funding in European Member States and/or DCs?
Is there likelihood of sustainability of the proposed approaches?
What is the research capability strengthening aspect of the proposal?
What is the training and mobility aspect of the proposal?
How is the integration of the DC partner(s) in the national setting?
How does the project fit in the international funding picture?

The evaluation of proposals is effectively done in four tranches:

I. Decision on eligibility by the Commission's Services based on partnership and documentation of the innovation and relevance of the project.
II. Scientific evaluation by selected experts.
III. Evaluation of the highly-rated proposals from the second tranche by a regional panel from developing countries
IV. Prioritisation of the highly-rated proposals based on coherence and complementarity with the Commission's existing research portfolio, partnership value, research capacity strengthening and training value and regional versus national relevance.

Following these procedures the Programme Committee consisting of the representatives of the Member States and associated countries, give an opinion on the proposals. Finally the Commission decides to implement the selected projects and activities.

References

EC (1996) Call for Proposals. *Official Journal of the European Communities* **39,** C75/31.

EC (1997) Call for Proposals. *Official Journal of the European Communities* **34,** C117/27.

ANNEX 3

LIST OF COLLABORATING INSTITUTES BY REGION/COUNTRIES

AFRICA

BENIN			Page:
Centre de Recherches Entomologiques	Cotonou	TS3*CT930236	31
Centre National Hospitalier et Universitaire	Cotonou	TS3*CT920068	57
O.C.C.G.E. Entomology Research Centre	Cotonou	IC18*CT970244	37
Université Nationale	Cotonou	TS3*CT920068	57
BURKINA FASO			
Centre de Rech. sur les Trypanosomoses Animales	Bobo-Dioulasso	TS3*CT920075	221
Centre National de Lutte contre le Paludisme	Ouagadoudou	IC18*CT970244	37
Centre National de Lutte contre le Paludisme	Ouagadougou	IC18*CT950015	91
Centre National de Lutte contre le Paludisme	Ouagadougou	IC18*CT970210	153
Programme d'Assistance Technique au CNLP	Ouagadougou	IC18*CT970210	153
CAMEROON			
Centre de Rech. Zootech. et Vétérinaires de Wakwa	Ngaoundere	TS3*CT920056	317
Institute of Medical Research	Kumba	IC18*CT970017	326
Medical Research Station	Kumba	TS3*CT910040	179
Medical Research Station	Kumba	TS3*CT920056	317
Ministry of Scientific and Technical Research	Yaounde	IC18*CT960112	209
OCEAC	Yaounde	TS3*CT920063	117
OCEAC	Yaounde	TS3*CT920084	123
OCEAC	Yaounde	IC18*CT960056	137
University Centre for Health Studies	Yaounde	TS3*CT930235	323
University of Buea	Buea	IC18*CT950026	307
University of Buea	Buea	TS3*CT920058	319
University of Buea	Buea	IC18*CT950017	326
Université Yaoundé	Yaoundé	IC18*CT970220	282
EGYPT			
Egyptian Organization for Vaccine Production	Cairo	IC18*CT970228	212
University of Alexandria	Alexandria	IC18*CT970228	212
University of Zagazig	Zagazig	IC18*CT970228	212
EQUATORIAL GUINEA			
Cooperacion Tecnica Espanola	Bata	TS3*CT910040	179

GABON			Page:
Centre International de Recherche Médicale	Franceville	TS3*CT920053	55, 89
Centre International de Recherche Médicale	Franceville	TS3*CT940346	55, 89
Centre International de Recherche Médicale	Franceville	TS3*CT920145	127
Centre International de Recherche Médicale	Franceville	IC18*CT950026	307
Centre International de Recherche Médicale	Franceville	TS3*CT940345	83
Dr. Albert Schweitzer Hospital	Lambarene	TS3*CT910040	179
Dr. Albert Schweitzer Hospital	Lambarene	IC18*CT970242	108
Université de Libreville	Libreville	TS3*CT930228	69
GHANA			
Noguchi Memorial Institute for Medical Research	Legon	IC18*CT950015	91
University of Ghana	Legon	IC18*CT970238	107
GUINEA-BISSAU			
Ministerio de Saúde Pública	Bissau	TS3*CT930224	129
IVORY COAST			
PRCT	Daloa	TS3*CT930246	239
KENYA			
Division of vector Borne Diseas	Nairobi	IC18*CT970237	214
Institute of Primate Research	Nairobi	IC18*CT960125	105
Institute of Primate Research	Nairobi	IC18*CT970212	172
Medical Research Institute	Nairobi	TS3*CT940296	167
Medical Research Institute	Nairobi	IC18*CT970227	143
Medical Research Institute	Nairobi	IC18*CT950015	91
Medical Research Institute	Nairobi	TS3*CT920066	189
Medical Research Institute	Nairobi	IC18*CT970237	214
Ministry of Health	Nairobi	TS3*CT920066	189
Ministry of Health	Nairobi	TS3*CT930237	197
Ministry of Health	Nairobi	IC18*CT970257	311
Ministry of Health/KEMRI	Nairobi	TS3*CT940330	204
University of Nairobi	Nairobi	IC18*CT970217	49
Wellcome Trust Kenya Medical	Kilagi	IC18*CT970272	208
MADAGASCAR			
Etablissement d'Enseignement Supérieur des Sciences	Antananarivo	TS3*CT930236	31
Université d'Antananarivo	Antananarivo	IC18*CT970244	37
CERMES	Niamey	IC18*CT970240	174
Ramse Programme	Antananarivo	IC18*CT970240	174
MALAWI			
University of Malawi	Blantyre	TS3*CT920083	147

ASIA

			Page:
BANGLADESH			
Interna tional Center for Diarrhoeal Research	Dhaka	TS3*CT930219	353
BURMA			
University of Yangon	Yangon	IC18*CT950016	93
CAMBODIA			
Ministry of Health	Phnom Penh	IC18*CT970211	35
CHINA			
Guangdong Shunde Institute of Laboratory Animals	Guangdong	IC18*CT960125	105
Institute of Parasitic Diseases	Shanghai	IC18*CT970241	181
Lanzhou Medical College	Lanzhou	TS3*CT940270	347
Nanjing Medical University	Nanjing	IC18*CT970243	177
Second Medical University	Shanghai	TS3*CT910040	179
Shanghai Institute of Animal Parasitology	Shanghai	TS3*CT920050	163
Shanghai Institute of Animal Parasitology	Shanghai	IC18*CT970243	177
Shanghai Second Medical University	Shanghai	IC18*CT950014	305
Xinjiang Medical College	Urumqi	TS3*CT940270	347
INDIA			
Banaras Hindu University	Varanasi	TS3*CT940271	325
Genetic Engineering and Biotechnology	New Delhi	TS3*CT930228	69
Indian Council for Medical Research	Bhubaneswar	TS3*CT920141	303
Indian Council for Medical Research	Bhubaneswar	IC18*CT970245	309
International Center for Genetic	New Delhi	IC18*CT960066	45
International Center for Genetic	New Delhi	IC18*CT950020	97
International Centre for Genetic	New Delhi	IC18*CT970223	141
International Centre for Genetic Engineering and Biotechnology (ICGEB) NII Campus	New Delhi	IC18*CT950022	103
INDONESIA			
Dr. Sardjito's General Hospital	Yogyakarta	TS3*CT930224	129
Hasanuddin University	Sulawesi Selatan	IC18*CT970245	309
University of Indonesia	Jakarta	TS3*CT910031	299
University of Indonesia	Atmadja	IC18*CT950014	305
LAOS			
Ministry of Health	Vientiane	IC18*CT970211	35
LEBANON			
American University of Beirut	Beirut	IC18*CT970256	290
OCCUPIED TERRITORIES			
Hebrew University Hadassah Medical School	Jerusalem	IC18*CT950023	273

EUROPE

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Institut Pasteur	Paris	TS3*CT940272	74
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Institut Pasteur	Paris	IC18*CT950021	101
Institut Pasteur	Paris	IC18*CT960071	47
Institut Pasteur	Lille	TS3*CT940317	81
Institut Pasteur	Lille	TS3*CT910030	158
Institut Pasteur	Lille	TS3*CT920118	165
Institut Pasteur	Lille	TS3*CT940303	171
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1. HIV/AIDS

HIV & Sexually Transmitted Diseases

*TS3*CT910022*
Studies on maternal and neonatal morbidity due to sexually transmitted diseases.

*TS3*CT920051*
A longitudinal epidemiological study of HIV-2 infection in Guinea-Bissau: transmission, disease development, immunology and prevention.

*TS3*CT920059*
Role of placenta in transmission of HIV infection from mother to child.

*TS3*CT920122*
Studies of the epidemiology of HIV infection and other sexually transmitted diseases in Mwanza region, Tanzania.

*TS3*CT930238*
Intervention study of sexually transmitted diseases and HIV infection among female prostitutes in Abidjan, Ivory Coast.

*TS3*CT930241*
The well-women clinic: studies on women's reproductive health.

IC18CT960050
A study on the role of HIV-1 genotypes on the epidemiology of HIV in Tanzania and Uganda.

IC18CT960110
Study of HIV-1 variability in Cameroon and Gabon. Implications for vaccine and antiviral intervention strategies.

IC18CT960114
Multicenter study on factors determining differential spread of HIV infection in African towns.

IC18CT970216
Virological and molecular characteristics of HIV strains in Africa: the impact of genetic subtypes on the pathogenesis and prognosis of HIV infection.

IC18CT970246
Study of HIV-1 variability in Cameroon and Gabon. Implications for vaccine and antiviral intervention strategies.

IC18CT970255
Development of a HIV candidate vaccine for phase I and II clinical trials in China.

HIV and Tuberculosis

*TS3*CT910033*
The application of DNA fingerprinting to the pathogenesis of HIV-related tuberculosis in Africa.

*TS3*CT910034*
Characterization of mycobacteria from HIV endemic area (Tanzania).

*TS3*CT930254*
Detrimental effects of TB on natural history of HIV infection: viral load, immune function and apoptosis.

IC18CT960083
Tuberculosis control in an urban area with high TB/HIV prevalence.

2. Mycobacteria

*TS3*CT910036*
A comprehensive investigation of advanced methods in molecular biology for the detection of *M. tuberculosis and M. leprae.*

*TS3*CT920062*
Mucosal immunity and leprosy.

*TS3*CT920099*
Immunological and molecular analysis of lymphocytes and antigens involved in leprosy (neuritic) lesions.

*TS3*CT920154*
DNA typing of *M. tuberculosis:* epidemiology and evaluation of chemotherapy.

*TS3*CT930243*
Rapid detection of multidrug-resistant mycobacteria.

*TS3*CT940001*
Approaches to the diagnosis of active tuberculosis based on the detection of specific mycobacterial components in the urine.

*TS3*CT940299*
Immune recognition of a novel 45KDA antigen, specific to *mycobacterium leprae*, and evaluation as a potential vaccine candidate.

*TS3*CT940304*
Peptides for the early detection and vaccination against tuberculosis and leprosy.

*TS3*CT940313*
Development of a new vaccine against *mycobacterium tuberculosis.*

IC18CT960047
Mucosal immunity of leprosy (MILEP2 study) definition of leprosy transmission and protection within an endemic leprosy population.

IC18CT960060
A mouse model for latent tuberculosis and prevention of reactivation of the disease.

IC18CT970236
Identification of protective immune responses to pathogenic mycobacteria.

IC18CT970253
The pathogenesis of tuberculosis; growth rate regulation and ribosome synthesis.

IC18CT970254
Development of tuberculosis vaccine with consistent efficacy in different regions of the world.

3. Hemorrhagic Fevers

*TS3*CT920067*
Diagnosis and control of Crimean-Congo Hemorrhagic Fever (CCHF).

*TS3*CT940286*
Epidemiology of filovirus infections in Central African Republic: a risk factors study associated with subsistence activities.

*TS3*CT940290*
The use of recombinant proteins and synthetic peptides for diagnostics and elucidation of virulence markers in dengue virus infections.

4. Human Papillomavirus (HPV)

*TS3*CT940295*
Human papillomavirus (HPV) infections in the pathogenesis of oesophageal cancer in the high-risk areas of China.

IC18CT970234
Development of Human Papillomavirus (HPV) vaccines for prevention and treatment of cervical cancer.

5. Measles

*TS3*CT910002*
The long-term impact on morbidity and mortality of measles infection.

*TS3*CT940302*
Study of the immune disruption caused by measles and its association with clinical progress in Dhaka, Bangladesh.

IC18CT950011
Specific and non-targeted effects of a two-dose strategy with standard titre measles vaccine. Implications for immunization programmes.

IC18CT960054
Factors associated with the severity of measles and of delayed complications of measles.

IC18CT960116
Contribution to the elimination of measles from Africa.

6. Pneumococcal vaccine development

*TS3*CT920086*
Polyvalent streptococcus polysaccharide capsular vaccine.

IC18CT950025
Evaluation of respiratory vaccines for children in South East Asia: *streptococcus pheumoniae* (and Hib) conjugate vaccines in prevention of childhood pneumonia in developing countries.

IC18CT970219
Field studies of pneumococcal conjugate vaccines to prevent ari in South East Asia — effectiveness of an 11-valent vaccine; safety and immunogenicity of a 4-valent vaccine under development.

7. Diarrhoea

*TS3*CT920060*
Intervention against diarrhoeal diseases by improving case management.

*TS3*CT920064*
Epidemiological and molecular biological study of cholera transmission dynamics in the Horn of Africa: a multidisciplinary approach.

*TS3*CT930255*
Oral vaccine against cholera with "built-in" adjuvanticity.

*TS3*CT940292*
The use of controlled release microparticles as oral and parenteral vaccines against *Vibrio cholera*, *Escherichia coli* and *Bordetella pertussis*.

*TS3*CT940311*
Persistent diarrhoea in early childhood — a prospective community study.

*TS3*CT940327*
Development and testing of an oral B subunit — whole cell cholera vaccine: protection against both 01 and 0139 cholera.

IC18CT960027
Early events in rotavirus infection: role of viral proteins on particle internalization and membrane permeability.

IC18CT970231
Pathogenesis of *vibrio cholerae* infection and persistence dynamics in the Horn of Africa.

8. Diphtheria and Tetanus

IC18CT950012
Evaluation of diphtheria- and tetanus-toxin neutralizing antibody activity in human and animal sera.

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*TS3*CT930259*
Epidemiological clinical and sero-virological studies of hepatitis C in Gabon and Brazil.

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*TS3*CT910035*
Leptospirosis in the Caribbean: a study on short and long term pathological consequences.

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*TS3*CT910024*
Bites and stings by venomous animals in Brazil: clinical and laboratory investigations of envenoming and therapy.

*TS3*CT920081*
Thalassemia intermedia syndrome: molecular haematological, clinical and therapeutic studies.

*TS3*CT920142*
Nutritional influences on the emergence of high blood pressure and diabetes in black afro-origin populations in Cameroon, Jamaica, France AND England.

*TS3*CT930244*
Molecular mechanisms of genetic variability in the expression of major hemoglobinopathies: prognostic value of genetic factors and therapeutic perspectives.

IC18CT950024
Development of a vaccine against *helicobacter pylori.*

IC18CT960032
South American bites and stings programme.

IC18CT960132
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