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Annual Scientific Report 1991

International Laboratory
for Research on Animal Diseases

P.O. Box 30709 • Nairobi • Kenya

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PREFACE

This is the fifth *Annual Scientific Report* to be published since ILRAD decided to make available individual research summaries of the work conducted by ILRAD's scientists and collaborators specifically for the scientific community. ILRAD also continues to publish a separate document, the *Annual Report*, which now adopts a format more accessible to the general reader.

In 1991, following the general recommendations of invited management consultants, ILRAD began to change from programme and laboratory based management to a more clearly defined, project-based research management structure. This internal review process, which will be completed in 1992, has led to slight changes in the descriptors adopted for this year's report. The present report groups research summaries by programme and project and the project areas and their objectives are listed below.

THEILERIOSIS

During 1991, the Theileriosis Research Programme reorganized its studies into three areas, with a combination of the work on the antigens of all three major life cycle stages (sporozoites, schizonts and blood stages) being described under a single heading. The three new project areas and their objectives are described below:

TH1. Epidemiology

To increase knowledge of the epidemiology of bovine theileriosis and to introduce and evaluate improved immunological control methods in collaboration with national and international organizations.

TH2. Antigens

To identify antigens of *Theileria* and other tick-borne pathogens of cattle which might be used as components of vaccines, as diagnostic tools or for parasite identification.

TH3. Vaccine delivery

To develop vaccines against *Theileria* and other tick-borne pathogens of cattle based on characterized antigens and effective antigen delivery systems.

TRYPANOSOMIASIS

The Trypanosomiasis Research Programme underwent major organizational change in 1991, but the scientific abstracts are grouped under the three major project areas on which the programme was based at the beginning of the year.

TR1. Epidemiology and drug resistance

To develop a more precise understanding of the epidemiology of tsetse-transmitted and non-tsetse-transmitted trypanosomiasis so that national organizations can better apply currently available control measures, with specific emphasis on the assessment of mechanisms of resistance to the currently employed trypanocides.

TR2. Biology and biochemistry

To obtain knowledge of important facets of the biology and biochemistry of the major pathogenic trypanosome species relevant to developing improved control measures for trypanosomiasis. The emphasis is placed on the definition of antigens associated with the cell surface and the endocytotic pathway, and on the mechanisms governing differentiation and cell division in trypanosomes.

TR3. Immunology and host resistance

To determine the immunological and related mechanisms involved in the acquisition or expression of resistance to trypanosomiasis. Work concentrates on the immunology of cattle infected with pathogenic trypanosomes and the immunological and genetic basis of trypanotolerance as exemplified by N'Dama cattle.

EPIDEMIOLOGY AND SOCIOECONOMICS

The objectives of the Epidemiology and Socioeconomics Programme are:

- To determine the factors that govern the successful application of improved livestock disease control, particularly through immunization.
- To predict, through the generation of computer models, the likely impact of improved animal disease control in epidemiological, economic, social and environmental terms.
- To develop disease control strategies that are appropriate for given agro-ecological areas, farming systems, governments and peoples.

Interested readers are referred particularly to ILRAD's scientific publications which are listed in this volume. The number in parentheses indicates the ILRAD publication number, and single copies of these publications are available on request.

It is our hope that the published papers and the enclosed abstracts of the latest scientific work of ILRAD will inform and assist colleagues elsewhere undertaking biological and socioeconomic research for the better control of diseases of veterinary and human importance.

P.R. Gardiner
Information and Planning Officer

THEILERIOSIS

TH1.1 International Project

Scientists: T. Dolan, S. Morzaria, R. Bishop, A. Young, C. Munatswa*, L. Lynen†, A. Rabia§
Research Associates: J. Katende, P. Spooner, B. Sohanpal, F. Mwakima, K. Kanhai*
Technicians: J. Kiarie, R. Njamunggch, L. Juma

*Central Veterinary Laboratory, Harare, Zimbabwe
†Belgian Annual Disease Control Project, Chipata, Zambia
§Department of Veterinary Services, Zanzibar, Tanzania

ILRAD has continued to support regional and national programmes for tick-borne disease control concentrating particularly on Pemba and Unguja islands of Zanzibar (Tanzania), Zambia and Zimbabwe. In all three countries, significant progress has been made in implementing immunization against theileriosis. Immunization has been applied in smallholder improved cattle on Zanzibar where both cows and calves have been immunized in a programme designed by the government and implemented jointly with donor-funded projects. A socioeconomic element has been introduced to the programme with a Zanzibar staff member trained at ILRAD and in Zimbabwe (with IDRC support). The donor support on Pemba (Ireland) and Unguja (FAO) is likely to end in 1992 but a sustainable programme may be in place by then.

In Zambia, a Belgium-funded project with local counterpart support has immunized over 50,000 indigenous cattle in an epidemic situation using a locally isolated stock of *Theileria parva*. Detailed epidemiological studies are being carried out and ILRAD is supporting this project with parasite characterization, development of models and provision of diagnostic reagents.

The problem of theileriosis in Zimbabwe differs from other countries of the region in that there is a fairly low mortality to cattle-derived infections (approx. 2000 p.a.) and sporadic deaths from buffalo-derived infections. A joint government/FAO project has completed a successful field immunization trial using a Zimbabwe isolate on two commercial farms involving 500 cattle. In addition some isolates have been made from buffalo-derived parasites to provide an immunizing stock to complement the cattle-derived parasite should the government decide to use immunization as a disease control measure. Over 40 cell lines have been sent to ILRAD and monoclonal antibody profiles and DNA probe analyses of about half of these isolates have been completed. The *T. parva* cattle-derived parasites (immunizing stock and field isolates) show a marked homogeneity. Isolates of *T. taurotragi* have also been identified. The project interacts closely with the ILRAD Epidemiology and Socioeconomics Programme in its activities in Zimbabwe.

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TH1.2 Selection and *in vivo* characterization of important *Theileria parva* stocks

Scientists: S. Morzaria, T. Dolan, R. Bishop
Research Associate: P. Spooner
Technicians: J. Kiarie, R. Njamunggeh, B. Sohanpal, L. Juma

Following the development of the infection and treatment method of immunization against *Theileria parva* and field exposure studies of immunized cattle, it was shown that different immunological types of *T. parva* existed. In attempts to identify the spectrum of different immunological types of *T. parva*, ILRAD and other institutes, in collaboration with national agricultural research services in Kenya and elsewhere, have isolated and characterized a large number of stocks from different geographical regions.

Until recently, various laboratories and institutes have been using these stocks for research purposes and have referred to them without regard to their origin and passage number. This had made comparison and sometimes interpretation of results from different laboratories difficult. In order to rationalize the use of these isolates we have selected 6 *T. parva* stocks of defined characteristics based mainly on their geographical origin and *in vivo* cross-immunity studies. The stocks selected are named Muguga, Marikebuni, Mariakani, Uganda, Boleni and buffalo-derived 7014.

The Muguga stock has been in use for over 30 years and most of the studies on the biology, immunology and the development of the infection and treatment immunization method have been performed using this parasite. This is the type species and the basis for comparison with other isolates. The Marikebuni and Mariakani stocks were originally isolated from the Kenyan Coast Province where buffalo-derived theileriosis does not occur. Both parasites have interesting immunological properties; the Marikebuni stock protects against 16 different isolates of *T. parva*. The Mariakani stock is the only stock known to break through the Boleni stock (see below).

The Uganda and Boleni stocks are parasites isolated from countries and from different epidemiological sites outside Kenya. The Uganda stock was isolated from an area in western Uganda where ECF is hyperendemic and the tick/parasite challenge is present throughout the year. The Boleni stock was isolated from an area in Zimbabwe where the disease is endemically unstable and seasonal. This parasite stock protects against several *T. parva* stocks from Kenya.

Limited cross-immunity studies with 5 stocks, Marikebuni, Muguga, Mariakani, Boleni and Uganda, revealed the following immunological properties: the Marikebuni stock protects against the 4 other stocks; the Muguga stock protects against the Mariakani stock but not against the Marikebuni stock; and the Boleni stock protects against the Marikebuni stock but not the Mariakani stock.

In vitro characterization of these parasites using various monoclonal antibodies and DNA probes have been completed. These 6 well characterized stocks of *T. parva* represent a unique research resource for studies of the biology and immunology of the parasite.

TH1.3 Selection of parasite stocks and determination of parasite dose for evaluation of the protective potential of sporozoite and schizont antigens

Scientists: T. Dolan, S. Morzaria, G. Gettinby*, A. Musoke, A. Young
Research Associates: F. Mwakima, P. Spooner, J. Scott
Technicians: J. Kiarie, S. Mwaura

*University of Strathclyde, Scotland

The *Theileria parva* (Muguga) bulk stabilate, 3087, is a major research resource providing most of the sporozoites being used in ILRAD's research on the antigens of *T. parva*. It is an important component in the development of cloning, characterization and serological techniques for *T. parva* and is the parasite being used in attempting to identify the factors influencing the infection rate in ticks. Infectivity, parasitological, clinical and pathological data have been collected over three years for 123 Boran cattle infected with different doses of the stabilate. One ml of undiluted, or 1:10 or 1:20 dilutions of the stabilate produce potentially lethal infection in all cattle so far tested. The stabilate has an estimated LD_{68} at a dilution of 1:100. Cattle suffering more prolonged reactions develop anaemia which may be profound (PCV of 14%). Small numbers of naturally recovered animals have been shown to be partially immune to challenge with the heterologous stock, *T. parva* Marikebuni (3014). This Marikebuni stock is also being used in immunological, characterization and titration studies. It has broad immunizing capacity (against 16 different stocks in cross-immunity studies) and is being used for immunization in Kenya.

Critical to the assessment of any potential vaccine is the quality of protection it provides. Preliminary predictions of vaccine efficacy can be obtained using stabilate challenge, and such challenges have been used extensively in the past in assessing the infection and treatment method of immunization. In using tick challenge, lethal challenges are readily provided from laboratory-infected ticks in which infection rates are frequently more than tenfold greater than field infections. To avoid overwhelming challenge and provide a challenge likely to resemble that in the field, data from a sample of laboratory infections in ticks infected with stabilate 3087 have been compiled. The data has been used in writing a computer programme to help determine the number of ticks, of known infection, which should be applied to particular numbers of cattle, so that a predictable number of infected ticks or infected acini are included in the challenge. Data on the development of infection and the severity of disease are already known from the stabilate titration studies described above. These can be used to assess the degree of immunity in instances in which immunization does not completely abrogate the challenge infection.

TH1.4 Studies on the preparation, handling and storage of *Theileria parva* sporozoite stabilates

Student: E.N. Kimbita
Supervisors: T. Dolan, S. Silayo*

*Sokoine University of Agriculture, Morogoro, Tanzania

Studies are directed towards the improvement in the preparation, handling and storage of *Theileria parva* sporozoite stabilates prepared from infected *Rhipicephalus appendiculatus* ticks. Using an infectivity titration assay *in vitro*, different media, cryoprotectants and freeze drying methods have been investigated. For preparation of sporozoites prior to freezing, L-15 medium gave the lowest end point titre. Glycerol at the standard concentration of 7.5% gave the best recovery and survival, and MEM medium with 3.5% BSA and 7.5% glycerol was the best mixture for sporozoite storage. The osmolarity of MEM and L-15 was increased five times by the addition of 7.5% glycerol. Sporozoites have not been successfully freeze-dried. Salivary glands from batches of ticks with low infection rates produced erratic infections *in vitro* even when the total numbers of infected acini used equalled that of highly infected batches. In experiments in which highly infected salivary glands were diluted with similarly treated, uninfected glands, the erratic infectivity was shown to be due to salivary gland toxicity for the bovine lymphocytes.

TH1.5 Characterization of *Theileria parva* using monoclonal antibodies

Scientists: S. Morzaria, T. Dolan
Research Associate: P. Spooner
Technicians: R. Njamunggeh, L. Juma

Monoclonal antibodies (MAbs) reacting with the schizont stage of *Theileria parva* have continued to be used in a panel for characterization of isolates of *T. parva*. The panel has been improved by the addition of MAb 23 which reacts specifically with *T. taurotragi* schizonts and permits separation of this species from *T. parva*.

The main use of the panel has been to monitor experimental infections and the efficacy of individual steps in the cloning of *T. parva* stocks. Also, the panel has been used to characterize field isolates from within Kenya and from collaborative projects with regional programmes outside Kenya. In particular, several isolates have been received from different locations in Zimbabwe. The majority of these isolates were found to have MAb profiles similar to the Boleni stock of *T. parva* with a few isolates characterized as *T. taurotragi*.

TH1.6 Isolation and characterization of polymorphic markers from *Theileria parva*

Scientists: R. Bishop, S. Morzaria, B. Allsopp*
Research Associates: E. Gobright, P. Spooner
Technicians: B. Sohanpal, T. Ndolo, J. Kiarie

*Veterinary Research Institute, Onderstepoort, South Africa

The repetitive DNA sequence TPR1 isolated from *Theileria parva* has proved useful for characterization, revealing extensive restriction fragment length polymorphisms (RFLPs) between the DNAs of most *T. parva* stocks. Hybridization of the TPR1 probe to DNAs isolated from cloned *T. parva*-infected lymphocyte lines derived from infections using a single *T. parva* stock has revealed the presence of several genetically heterogeneous components within a number of cattle-derived *T. parva* stocks.

Synthetic oligonucleotides derived from TPR1 have been developed and used to differentiate the Muguga and Uganda stocks on a +/– basis. These oligonucleotides have been used to screen polymerase chain reaction (PCR) amplified sporozoite DNA for potential recombinants between the two stocks arising from a sexual cycle. An oligonucleotide has also been developed which differentiates the Marikebuni stock, which is currently being used for immunization trials in Kenya, from five other stocks on a +/– basis. This may prove a valuable reagent for assessing whether transfer of the immunizing stock into naive ticks occurs after immunization by infection and treatment.

Probing Southern blots of *Sfi*I digested DNAs from the Muguga and Uganda stocks with TPR1 probes suggests that the TPR1 repetitive sequences from which the +/– markers are derived may be present on different chromosomes in these two stocks. These oligonucleotides may therefore represent markers for independent assortment of chromosomes in crosses between these stocks.

The use of *T. parva* ribosomal DNA probes for parasite characterization has been evaluated. Probing of *Eco*RI digested DNA with a *T. parva* small ribosomal subunit RNA gene probe splits *T. parva* stocks into two groups based on polymorphic restriction fragment patterns. Probing of DNA digested with *Acc*I and *Pvu*II reveals the existence of finer groupings among the stocks. The transcribed spacer region between the large and small ribosomal subunit genes has been PCR-amplified and sequenced from DNAs isolated from both the Uganda and Muguga stocks and large differences are apparent in the DNA sequence between the two stocks. It should therefore be possible to develop +/– markers for these two stocks derived from the ribosomal locus.

A *T. parva* telomeric DNA sequence has been cloned using a synthetic oligonucleotide based on a published *Plasmodium* telomeric sequence. When used to probe *T. parva* DNA the telomeric probe recognizes 7 or 8 *Eco*RI fragments in most *T. parva* stocks. The majority of these fragments are polymorphic in size between different stocks. Since it recognizes polymorphic restriction fragments situated at the ends of all 4 *T. parva* chromosomes, the telomeric probe can be used to examine independent assortment of chromosomes in recombinant parasites.

TH1.7 Identification and purification of tick stages of *Theileria parva*

Scientists: T. Imada*, A. Young, T. Dolan, S. Morzaria
Research Associate: F. Mwakima
Technicians: S. Mwaura, R. Njamunggeh, G. Njehia

*National Institute of Animal Health (NIAH), Tsukuba, Japan

Accurate identification of the different developing stages of *Theileria parva* in the tick *Rhipicephalus appendiculatus* is important in order to study the factors which control the infection rates in tick salivary glands. In preliminary studies experiments were designed to: 1) study the dynamics of parasite development in ticks; 2) develop methods of purifying tick stages of *T. parva*; and, 3) develop serological markers for the identification of the different tick stages.

Preliminary studies using the indirect fluorescent antibody test showed that polyclonal sera from cattle recovered from ECF, and monoclonal antibodies specific for sporozoite, schizont, merozoite and piroplasm stages of *T. parva* did not react against gametes, zygotes or kinete stages from the tick. This study provided evidence that *T. parva* parasites from ticks have specific antigens different from those of the stages of *T. parva* from cattle.

The development of kinetes was studied in engorged nymphs and moulting and feeding adult ticks. The highest number of kinetes were harvested from ticks between 19 and 25 days post-repletion. There was no relationship between the number of kinetes produced and the parasitaemia of cattle on which the nymphal batches had completed engorgement. There appeared, however, to be a direct relationship between the number of kinetes detected and the number of salivary gland acini infected in the same batch of ticks.

Using a Percoll-based purification technique, 4×10^4 kinetes were obtained from 400 engorged nymphal ticks but these kinetes were contaminated with tick cells and cellular debris. Attempts at further purification of the kinetes were unsuccessful.

Development of gametocytes in the gut of the engorged nymphal ticks which had fed on *T. parva*-infected cattle was also investigated. It was found that the best day for harvesting gametocytes from replete nymphs was on day 3 or 4 after repletion. Less than 10% of ticks in any particular batch feeding on highly parasitaemic cattle developed large numbers of gametocytes. Using discontinuous density, Percoll gradient centrifugation, a highly enriched population of gametocytes was obtained, as confirmed by electron microscopy.

These studies have enabled the development of a method of obtaining large numbers of gametocytes from engorged nymphs and provide possibilities for generating MAbs specific for the stage. The kinete stage is difficult to purify and further studies are in progress to develop a more efficient method of purification.

TH1.8 Application of DNA technology to the identification of *Theileria* species

Scientists: R. Bishop, S. Morzaria, A. Young, B. Allsopp*
Research Associates: E. Gobright, P. Spooner
Technicians: B. Sohanpal, J. Kiarie

*Veterinary Research Institute, Onderstepoort, South Africa

Repetitive DNA probes which are species-specific when used under high stringency washing conditions have been isolated from *Theileria parva* and *Theileria mutans*. Both the *T. parva* and *T. mutans* probes are capable of detecting approximately 1 ng of parasite DNA which is considerably less than the reported sensitivities of repetitive DNA probes isolated from *Plasmodium* species (.01 ng). When used to probe slot-blotted DNA isolated from the blood of *T. parva* infected carrier cattle, the *T. parva* repetitive probe was not sufficiently sensitive in detecting *T. parva* DNA sequences, although the presence of these sequences was confirmed using polymerase chain reaction (PCR) amplification of DNA (see below). The *T. mutans* probe was capable of detecting parasite DNA when hybridized to whole blood DNA derived from some field infected cattle. A portion of the *T. mutans* probe has been sequenced and PCR primers for amplification of *T. mutans* DNA from carrier animals have been developed. The *T. parva* repetitive probe has been successfully used for detection of *T. parva* sporozoites by probing squash blots of dissected infected *Rhipicephalus appendiculatus* salivary glands. Hybridization of the *T. mutans* probe to Southern blots of *T. mutans* DNA digested by restriction enzymes reveals the presence of restriction fragment length polymorphisms (RFLPs) between the two available stocks. Interestingly a *T. parva* repetitive DNA sequence isolated from the *T. parva* Boleni stock has proved useful for characterization of *T. taurotragi* stocks and reveals RFLPs when hybridized to DNAs from different *T. taurotragi* stocks.

PCR amplification of *T. parva* DNA has been used for detection of *T. parva* carrier cattle. Two sets of primers, one derived from a *T. parva* Muguga TPR1 repetitive DNA sequence and the other from the p67 *T. parva* sporozoite antigen gene, were used to amplify parasite DNA. Initially, purified DNA of whole blood isolated from carrier cattle was used in these assays but subsequent amplification was obtained from minimally processed whole blood samples. Successful amplification was also obtained from blood samples which had previously been spotted onto nitrocellulose filters, and kept an ambient temperature for up to three weeks prior to amplification.

The use of probes which specifically hybridize to ribosomal RNA or ribosomal DNA has also been evaluated for *Theileria* species differentiation. Sequencing of the DNA of small subunit ribosomal RNA genes from different *Theileria* species has revealed the existence of species-specific regions. Synthetic oligonucleotides derived from these regions have been developed which are specific for *T. parva, T. mutans, T. taurotragi, T. annulata, T. buffeli* (Marula) and an unknown *Theileria* species isolated from buffalo. The oligonucleotides have been used to differentiate these *Theileria* species in a +/– manner by probing either ribosomal DNA, amplified from whole genomic DNA using the PCR, or parasite ribosomal RNA in schizont-infected lymphocytes. When ribosomal RNA was probed, *T. parva* and *T. taurotragi* were clearly discriminated and as few as 100 schizont-infected cells were detected. The

oligonucleotide probes have also been applied to detection of parasite ribosomal RNA in infected tick salivary glands. *T. parva* and *T. taurotragi* were differentiated in squashes of dissected, infected *Rhipicephalus appendiculatus* salivary glands. Application of the small subunit ribosomal probes to ticks, however, still requires optimization to improve sensitivity.

TH1.9 Characterization of a newly isolated *Theileria* of cattle from Marula, Kenya

Scientists: S. Morzaria, A. Young, T. Dolan, R. Bishop, M. Shaw, V. Nene, A. Kairo, S. Williamson*, S. Kawazu†, A. Lesan§, P. Ngumi§
Research Associate: F. Mwakima
Technicians: J. Kiarie, T. Ndolo

*Centre for Tropical Veterinary Medicine (CTVM), Edinburgh, Scotland
†National Institute of Animal Health (NIAH), Tsukuba, Japan
§Kenya Agricultural Research Institute (KARI), Muguga, Kenya

Some 'non-pathogenic' *Theileria* of cattle under certain conditions may cause disease. These *Theileria* also confound epidemiological studies as they commonly occur concurrently with *T. parva* and may cause serological cross-reactions. Thus it is important to identify these piroplasms and develop accurate diagnostic tests.

Recently in an East Coast fever immunization field trial on Marula ranch, Naivasha, Kenya, an animal was observed to have a high parasitaemia of piroplasms with an atypical morphology. The parasite, designated as *Theileria* Marula, was isolated by subinoculation of infected blood into cattle. The infection in experimental cattle was characterized by high parasitaemia and anaemia. The piroplasm-infected erythrocytes showed the presence of bars and veils. Following infection, cattle developed high antibody titres to piroplasms (1/640–1/2560) but low level (1/160) cross-reactivity was also observed with *T. mutans*, *T. parva* and *T. taurotragi* piroplasms in the indirect fluorescent antibody test. This, and the unusual vector requirement—the parasite could not be transmitted by *R. appendiculatus* and *A. variegatum* ticks—confirmed the need for a specific diagnostic test for the Marula parasite.

In an attempt to identify the species of the *Theileria* Marula parasite, DNA prepared from the piroplasm stage of the parasite was analysed using various *Theileria* species-specific probes and also compared with the DNAs of *T. parva*, *T. mutans*, *T. taurotragi*, *T. sergenti*, *T. annulata* and *T. buffeli*. These analyses showed that the Marula parasite is identical to *T. buffeli*. The rDNA probe generated from the Marula stock cross-hybridized with Australian *T. buffeli*, in confirmation of the above finding. It is suspected that this parasite is transmitted to cattle by *Haemaphysalis* spp. and experiments are in progress to attempt transmission of the Marula stock with *H. lechii*.

TH1.10 Development of antigen and antibody trapping ELISAs for tick-borne diseases

Scientists: A. Musoke, S. Morzaria, A. Young, V. Nene, T. Dolan, T. McGuire*, W. Jorgenson†, J. Ngeranwa§, S. Okuthe§, S. Williamson‡, J. Grootenhuis, A. Lesan‡, P. Ngumi‡
Research Associates: J. Katende, C. Nkonge, P. Spooner, F. Mwakima
Technicians: A.N. Maina, M. Omenya, L. Gichuru, A. Sore, J. Tangus

*Washington State University, Pullman, USA
†Tick Fever Research Centre, Wacol, Australia
§Wildlife Diseases Research Project (WDRP), Kabete, Kenya
‡Kenya Agricultural Research Institute (KARI), Muguga, Kenya

In planning control strategies against *Theileria parva* and other tick-borne diseases, accurate data on prevalence and incidence are necessary. Currently these data are difficult to produce because of the lack of specific and sensitive, field applicable serological tests. The aim of this project is to develop improved ELISAs with higher specificities and sensitivities than the currently available tests for the major tick-borne pathogens.

Antigen and antibody capture ELISAs have already been developed for *Theileria mutans* based on a piroplasm-specific 32 kDa antigen. The tests detect circulating antigen, immune complexes and antibodies in acute and chronic infections and carrier cattle. Laboratory validation studies have shown that the tests are specific for *T. mutans* and no cross-reactivities have been detected with other *Theileria* species *(T. parva, T. annulata, T. taurotragi, T. buffeli and T. orientalis)*, or with *Babesia bigemina, B. bovis* and *Anaplasma* species. Limited field studies have shown that the test is superior in sensitivity and specificity to the indirect fluorescent antibody (IFA) test. The gene encoding the 32 kDa antigen of *T. mutans* has been cloned and is currently being sequenced.

A conserved antigen of 200 kDa has proved to be more useful than the previously selected candidate diagnostic antigen of 44 kDa of *B. bigemina*. Monoclonal antibodies recognizing different epitopes on the conserved 200 kDa antigen of *B. bigemina* have been generated. Antigen capture ELISA detects infection in cattle as early as 3 days after inoculation of parasites, and continues to identify circulating antigen in acute and chronic infections and in carrier cattle. Antibody detection ELISA, using the affinity purified 200 kDa antigen, provides sensitivity higher than the currently available IFA test. Both the antigen and antibody detection ELISAs do not show cross-reactivity with other major tick-borne pathogens (see above).

Two additional *Anaplasma marginale* specific antigens of 70 kDa and 28 kDa molecular weights have been identified which appear to be more useful than the previously identified 52 kDa antigen. Monoclonal antibodies against both these antigens provide highly specific antigen capture ELISA, and the affinity purified antigens have proved to be superior to the currently available agglutination test in detecting acute and chronic infections and carrier animals.

Studies are under way to identify appropriate, parasite-specific diagnostic antigens for *T. parva, T. taurotragi* and *T. buffeli*. Laboratory validation of the ELISAs for

each of these parasites will involve testing for specificity using defined positive and negative control sera from experimental and field infections from various known stocks of parasites from different geographical regions. Additionally, the sensitivity of the antigen capture ELISAs will be compared with that of DNA-based oligonucleotide probes used with PCR-amplified products.

TH1.11 A restriction map of the *Theileria parva* genome

Scientists: S. Morzaria, J. Young*
Technicians: T. Ndolo, J. Kiarie

*Institute for Animal Health, Agricultural and Food Research Council (AFRC), Compton, UK

The objectives for the construction of a physical map of the *Theileria parva* genome are to define the basis of *Sfi*I polymorphisms observed in different stocks, identify deleted regions for stock characterization, locate antigen and other genes on the map and provide a set of markers for studies of sexual recombination. The studies will enable identification of the polymorphic regions of the parasite genome and will assist in defining and characterizing different immunological stocks of *T. parva* for use in 'infection and treatment', and other forms of immunization.

The mapping strategy followed was to clone short DNA fragments spanning the *Sfi*I sites, called *Sfi* linking clones, and use them as probes to reveal which *Sfi*I fragments are directly linked in the genome. An *Sfi*I linking library of approximately 70,000 recombinants was constructed from 4–6 kb partially *Sau*3A digested *T. parva* Muguga DNA. Analysis of the minipreps from 350 clones from the library revealed that 199 had DNA fragments of the appropriate size. Further analysis by end-labelling and acrylamide and agarose gel separation of *Sau*3A and *Sau*3A/*Sfi*I double digests showed 123 clones had *Sau3A* fragments bearing *Sfi*I sites. These were sorted into 21 unique groups each representing one *Sfi*I site in the genome. The linking clones were used in three kinds of analysis. Hybridization to blots of *Sfi*I fragments was used to establish linkage between *Sfi*I fragments directly. The clones were then used as specific markers to assign *Sfi*I fragments to separated chromosomes and *Not*I fragments. Finally, two clones were used for mapping partial digestion ladders. The telomeres were identified using an oligonucleotide probe to a *Plasmodium berghei* telomeric fragment.

The complete genomic map reveals 29 *Sfi*I and 4 *Not*I sites in the 10 megabase pair genome of *T. parva* Muguga. The map reveals 4 chromosomes. Six antigen genes, the TpR sequence, ribosomal DNA and 6 uncharacterized cDNAs from *T. parva* schizonts have been located on the map.

TH1.12 Evidence for a sexual cycle in *Theileria parva*

Scientists: S. Morzaria, J. Young*, R. Bishop, A. Young, T. Dolan, H. Mehlhorn†
Student: E. Njenga†
Research Associates: P. Spooner, F. Mwakima
Technicians: B. Sohanpal, J. Kiarie, T. Ndolo

*Institute for Animal Health, Agricultural and Food Research Council (AFRC), Compton, UK
†Ruhr-University Bochum, Bochum, Germany

Members of the phylum Apicomplexa are generally known to undergo a sexual cycle and in *Plasmodium* spp. evidence for genetic recombination as the major cause of polymorphism has been provided. In *Theileria parva*, a sexual cycle has been presumed to occur and differentiation of piroplasms into morphologically distinct, macro- and micro-gametes has been described. However there is no direct genetic evidence of this phenomenon. It is important to establish if a sexual cycle occurs in *T. parva* and then to ascertain if the extensive polymorphism shown by this parasite is produced by sexual recombination. This knowledge is important in planning immunization strategies against East Coast fever.

Two *T. parva* stocks, Muguga and Uganda, have been selected for the study of a sexual cycle. The selection was principally based on the availability of +/– DNA markers and distinct monoclonal antibody (MAb) profiles. The developmental cycle of both stocks show that, following the administration to cattle of a standard sporozoite dose, they reach their peak piroplasm parasitaemia at around the same time, thus providing the opportunity for ticks to be infected equally with both parasites.

Two cattle were each infected with mixed Uganda and Muguga stocks of *T. parva* while 2 control animals were each infected with either Uganda or Muguga stock. *R. appendiculatus* nymphs were applied to the infected animals to feed throughout the period of piroplasm parasitaemia and the moulted adult ticks from each animal were examined for sporozoite infection.

Sporozoites from a single infected acinus were assumed to have been derived from a single kinete. Aliquots of such sporozoites were used to infect bovine lymphocytes *in vitro*, and for the preparation of DNA. The DNA was amplified by the polymerase chain reaction with *T. parva* specific primers. Sporozoites showing mixed genotypes, and positive hybridization with the Muguga and Uganda specific probes, were considered as putative recombinants. These were cultured and characterized further with MAbs and various DNA probes.

Eight out of 66 dissected ticks, derived from cattle infected with mixed Muguga and Uganda parasites, were each found to have a single infected acinus. Sporozoites from 3 of these showed a mixed genotype and only one of these grew in lymphocyte culture. On further characterization of the cultured parasite, the MAb profile and the DNA showed a Muguga type of phenotype, while probing with the telomeric probe showed that the *EcoRI* fragments bearing telomeres were of mixed Muguga and Uganda type—thus providing evidence of independent assortment of chromosomes in the recombinant parasite. This is the first time evidence for the presence of a sexual cycle has been demonstrated. The presence of a sexual cycle is further supported by

the recent confirmation that the gametes and the zygotes of *T. parva* in the tick gut are haploid and diploid respectively.

TH1.13 Entry and establishment of *Theileria parva* sporozoites in bovine host cells

Scientists: M. Shaw, L. Tilney*, A. Musoke, A. Young
Research Associates: C. Wells, C. Nkonge
Technicians: P. Theuri, J. Gachanja

*Department of Biology, University of Pennsylvania, Philadelphia, USA

Previous studies have shown that the process of *Theileria parva* sporozoite entry into susceptible bovine lymphocytes involves a defined series of discrete but contiguous events.

Although much information is available on the range of bovine cells in which *T. parva* will establish infection, little is known about the diversity of host cells into which sporozoites may enter without being able to establish and develop. As part of our efforts directed at the elucidation of the mechanism(s) involved in sporozoite entry, we have examined the ability of sporozoites to enter a range of potential host cells. The range of host cells used was chosen to correspond to those cells known to be present around the tick attachment site. The results of these experiments showed that sporozoites recognize and enter only lymphocytes and to varying degrees certain types of macrophages, in particular afferent lymph veiled cells (ALVC) The sporozoites enter ALVC in a manner similar to entry into lymphocytes and only on rare occasions were sporozoites found to be phagocytosed by the macrophages. Although sporozoites enter ALVC and develop into early multinucleate schizonts, it has not been possible to obtain transformed cell lines.

While *T. parva* sporozoites only bind to and invade a very limited range of bovine cells, the numbers of infectible cells within a population of lymphocytes can be increased by components in tick saliva and by a number of reagents previously implicated in the process of cellular or immunological activation (e.g. IL-2, phorbol esters).

As part of continuing efforts to define the sporozoite and host cell surface molecules involved in the entry process we have examined the inhibitory effects of a number of monoclonal antibodies (MAbs) reactive with sporozoite and host surface molecules. So far MAbs against both native and recombinant sporozoite p67, and against p85 molecules inhibit, in a dose-dependent manner, sporozoite entry. Results using MAbs against lymphocyte surface molecules, with the exception of MAbs reactive with MHC class I, have not been conclusive. Studies to try to identify more host cell surface molecules involved in sporozoite entry are continuing.

The possible role of Ca^{2+} in sporozoite entry has been examined. Depletion of Ca^{2+} from the external environment had no inhibitory effect on sporozoite entry. Inhibitors of Ca^{2+} channels and calmodulin antagonists had a dose-dependent, reversible inhibitory effect. Depletion of intra-sporozoite Ca^{2+} levels also had an inhibitory effect. Studies are now in progress to try to identify, by cytochemical means, the intra-sporozoite site(s) of Ca^{2+} storage.

TH1.14 A linear 7.1 kbp DNA molecule encodes a subset of *Theileria parva* mitochondrial proteins

Scientists: V. Nene, M. Shaw, S. Morzaria, A. Fairlamb*
Research Associate: E. Gobright
Technicians: S. Wanyoni, T. Ndolo
Student: A. Kairo
Supervisors: V. Nene, A. Fairlamb*

*Department of Protozoology, London School of Hygiene and Tropical Medicine, UK

Analysis of total piroplasm *Theileria parva* genomic DNA on agarose gels reveals the presence of an 'extra-chromosomal' genetic element that migrates at 7.1 kbp. This element is a double stranded DNA molecule which hybridizes only to itself in Southern blot analysis of total genomic DNA, suggesting that the element does not represent amplification of chromosomal sequences. The element appears to be linear in structure. This has been confirmed by demonstrating that the element is susceptible to *Bal*31 digestion.

Five other species of *Theileria* and two species of *Babesia* contain a similar size DNA molecule to the *T. parva* element. There are distinct restriction fragment length polymorphisms between the elements which makes the *T. parva* element a useful diagnostic probe for mixed parasite infections.

About 6 kbp of the *T. parva* element has been cloned and sequenced. The element has the potential to code for cytochrome b and polypeptide I of cytochrome c oxidase. A third gene has a limited sequence similarity with polypeptide III of cytochrome oxidase. In addition there are short, dispersed stretches of DNA sequences which have a high degree of sequence identity with rRNA sequences. These split rRNA sequences have the potential to base pair with each other to constitute some of the core regions which are conserved features of large and small subunit rRNA molecules. The finding of genes coding for mitochondrial proteins and perhaps functionally split rRNA genes on the 7.1 kbp DNA element suggests that this DNA is mitochondrial in origin. The features of the *T. parva* DNA are reminiscent of the mitochondrial genome of *Chlamydomonas reinhardtii*, a unicellular green alga.

TH1.15 The biology of the TPR multicopy gene family of *Theileria parva*

Scientists: R. Bishop, S. Morzaria, A. Musoke, B. Allsopp*
Research Associates: C. Nkonge, E. Gobright, C. Wells
Technician: B. Sohanpal

*Veterinary Research Institute, Onderstepoort, South Africa

Sequencing of *Theileria parva* Muguga genomic DNA has revealed the existence of a family of partially repeated open reading frames (ORFs) arranged in tandem. The unit repeating elements within these ORFs in *T. parva* Muguga have been designated TPR1, TPR2 and TPR3. The DNA sequences of these ORFs are only 70% identical

between different *T. parva* stocks suggesting that the proteins which they potentially encode are evolving rapidly. The TPR gene family has the appearance of a system designed to generate diversity and the organization is reminiscent of vertebrate immunoglobulin or troponin T genes.

Attempts have been made to express portions of the ORFs identified in the *T. parva* genomic DNA sequences, in *Escherichia coli* plasmid expression vectors, in order to raise antisera which can be used to identify TPR proteins on Western blots and in immuno-electron microscopy. Two hundred and fifty amino acids at the carboxyl terminus of an ORF identified in *T. parva* Boleni and 130 amino acids at the carboxyl terminus of an ORF identified in *T. parva* Muguga have been expressed as fusions with the *Schistosoma japonicum* glutathione S-transferase (Sj26) using the pGEX expression vector. The Sj26:TPR fusion proteins, which were unstable, were used to immunize rats. The resulting antisera recognize a protein of approximately 70 kDa molecular weight in uninfected *Rhipicephalus appendiculatus* tick salivary glands on Western blots. The anti-Sj26:TPR sera does not consistently recognize any proteins in schizonts, piroplasms or sporozoites of *T. parva* on Western blots. Four hundred and seventy amino acids at the carboxyl terminus of a TPR ORF identified in the *T. parva* Muguga stock have been expressed fused to the N-terminal 80 amino acids of NS1 (an influenza non-structural protein) using the PMG1 expression vector. The levels of expression achieved with the NS1 construct are however low and inconsistent and this fusion protein will be difficult to use for immunizations.

Probing of RNA blots with TPR clones reveals the presence of a 3.5 kilobase transcript in the piroplasm stage but not in schizonts. The size of this transcript is considerably larger than the largest ORF identified in the genomic DNA sequences (2.4 kb). Five piroplasm cDNA clones homologous to TPR genomic DNA clones have been isolated. Two TPR-homologous cDNA clones of approximately 1.3 kb were isolated by screening a piroplasm cDNA library and three additional clones of approximately 0.8 kb were obtained by polymerase chain reaction amplification of piroplasm cDNA using one TPR primer and one oligo(dT) 'adaptor' primer. DNA sequencing demonstrates the presence of 4 different 3′ non-translated sequences among the 5 cDNA clones. The two cDNA clones sharing homology in the 3′ non-translated region exhibited some sequence differences within the ORF. There are thus at least 5 different TPR transcripts derived from piroplasms present in a single infected animal, although they do not necessarily originate from within a single parasite genome.

TH1.16 Identification and characterization of *Theileria parva* multidrug resistant gene(s)

Student: M. Kibe
Supervisor: O. ole-MoiYoi

Drug resistance constitutes a serious obstacle to effective chemotherapeutic treatment. Multidrug resistance (MDR) in some protozoan parasites, as in tumour cells, is associated with a group of membrane proteins known as P-glycoproteins, which are encoded by a small group of closely related genes referred to as MDR genes. This study aims

to look at the role of P-glycoproteins in the transport of drugs or molecules in *Theileria parva*.

Oligonucleotides homologous to the nucleotide binding domains of a *Plasmodium falciparum* (MDR) gene were used to screen, at low stringency, genomic libraries of *Theileria parva*. A 750 bp fragment was isolated and, on sequencing, two nucleotide binding domains were found. There was also a high degree of homology to the nucleotide-binding domains of other P-glycoproteins from prokaryotic and higher eukaryotic organisms.

The 750 bp fragment was used to screen for a large (5.7 kb) *Sal*I genomic fragment. Partial sequence of a 4450 bp region of this large fragment revealed two nucleotide binding domains but no large open reading frame. When the 750 bp fragment was used to screen cDNA libraries made from *T. parva* schizont-infected cells, several clones were identified. Partial sequence of some of these clones suggests the presence of two different types of transcripts. One clone of 2.2 kb was not full length but on further screening, two overlapping clones were isolated. Partial sequence of the 2.2 kb clone and the two overlapping clones reveals an open reading frame of 594 amino acids, predicting a protein of about 80 kDa. Another 3.0 kb clone, which appears to be full-length, was also isolated. Differences in the partial sequences were the presence, in the 3.0 kb clone, of a 57 bp insertion and GC rich 5′ untranslated region.

Comparison of the sequence of the 2.2 kb clone with the 4450 bp genomic DNA fragment, showed the presence of seven introns in the genomic DNA. On the other hand, Southern blots of genomic DNA cut with several restriction enzymes and probed with 2.2 or 3.0 kb fragments did not show any difference in the hybridization patterns.

These results suggest that *T. parva* has an MDR-like gene which is not duplicated as is the case in higher eukaryotes. However, the organism produces two MDR gene transcripts from the same MDR gene locus possibly by an alternative splicing mechanism. Work is in progress to characterize MDR-like genes in other *T. parva* stocks to complete sequencing the genomic and cDNA clones and to produce fusion protein for a generation of antibodies which will be used to localize the P-glycoprotein in infected cells by immunoelectron microscopy.

TH2.1 Immune CD4+ T-cells specific for *Theileria parva*-infected lymphocytes recognize a 24 kDa protein

Scientists: D. Grab, C. Baldwin*, W. Brown†, E. Innes§, J. Lonsdale-Eccles
Technician: Y. Verjee

*Department of Microbiology, The Ohio State University, Columbus, USA
†Department of Veterinary Microbiology and Parasitology, Texas A & M University, College Station, USA
§Moredun Research Institute, Edinburgh, Scotland

Theileria parva is a protozoan parasite that infects and transforms bovine lymphocytes. Here we report the partial purification of a *T. parva*-specific protein from infected lymphocytes which is recognized by CD4+ T-cell clones derived from immune cattle. *Theileria parva*-infected lymphocytes were homogenized in Dulbecco's phosphate buffered saline in the presence of protease inhibitors. The antigen was purified from a post-microsomal supernatant using a combination of DEAE-cellulose and hydroxylapatite column chromatography. After labelling with ^{125}I, the partially purified antigen preparation was found to contain eight to ten proteins by SDS-PAGE analysis. This preparation was subjected to chromatography in phosphate buffered saline on HPLC TSK-250/125 columns coupled in tandem. A radiolabelled protein of Mr of 24 kDa correlated with antigenic activity.

TH2.2 Analysis of expression of the sporozoite p67 gene after transfection into mammalian cells

Scientists: P. Toye, V. Nene, A. Musoke
Research Associates: E. Gobright, C. Nkonge
Technicians: J. Nyanjui, D. Ngugi

We have used the gene encoding the sporozoite p67 antigen to study the expression of theilerial antigens in mammalian cells. Such studies are considered important for the application of transfection technology to analyse cytotoxic T-lymphocyte reactivity with parasite proteins.

COS cells transfected with a cDNA copy of the p67 gene reacted with several monoclonal antibodies specific for this antigen. The pattern of staining on fixed, permeabilized cells, plus the lack of reactivity with live cells, indicated that the molecule was not expressed on the surface of the transfected cells. The frequency of cells expressing the protein was maximal 2 to 3 days after transfection, and was about 20–40% of the cell population. Immunoblots of the transfected cells revealed that the antigen was expressed as a molecule of Mr ~ 89 kDa, which is larger than the major form of the protein in sporozoites but smaller than the protein expressed in mammalian cells by recombinant vaccinia viruses.

The genomic version of the p67 gene contains a 29-bp intron. We were interested to know whether mammalian cells could efficiently remove this intron. Transfection of a plasmid containing the genomic version of the p67 gene resulted in

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cells which reacted with anti-p67 monoclonal antibodies. However, immunoblot analysis of the transfectants indicated that the expressed product was of Mr ~ 54 kDa, which is expected if the intron was not excised. Thus it appears that COS cells failed to process the genomic version of the gene, which indicates that the use of genomic libraries for the screening assays may have serious limitations.

TH2.3 Use of transfection technology to identify antigens recognized by cytotoxic T lymphocytes from cattle immune to *Theileria parva* infection

Scientists: P. Toye, A. Bensaid, H. Clevers*, P. Wijngaard*, M. Metzelaar*
Research Associate: N. MacHugh
Technicians: J. Nyanjui, D. Ngugi, A. Kaushal

*Department of Clinical Immunology, Academic Hospital, Utrecht, Holland

The objective of this project is to identify, using cytotoxic T lymphocytes (CTL) from immune cattle, antigens of *Theileria parva* which can induce immunity in cattle. The technology for using CTL directly to identify their respective epitopes is still in the developmental stage. The essence of this technology involves transfection of a library of *Theileria parva* genes into a mammalian cell line expressing bovine class I MHC molecules, identification of relevant gene product with CTL from immune cattle and recovery of the gene. This work comprises two components, a) the generation of a suitable recipient cell line and, b) the establishment of a feasible assay system.

a) Recipient cell line. Initially it was hoped that mouse L cells, permanently transfected with the parasite gene library, would prove a suitable screening reagent. Attempts to generate L cells permanently transfected with several *Theileria parva* genes, including that encoding the p67 sporozoite antigen, have failed to yield cells expressing detectable parasite proteins. However, it was observed that the p67 sporozoite antigen was expressed in transiently transfected COS cells. In order to use COS cells for screening by CTL, we have generated a COS cell line which permanently expresses the bovine class I MHC gene, KN104. This cell line was cloned by limiting dilution, and one clone has remained stable for KN104 gene expression during at least four months of continuous culture.

b) Assay System. We have examined the feasibility of using direct microcytotoxicity assays on a library of genes transfected into COS cells. As a schizont is thought to express between 5–10,000 gene products, the most practicable way of analysing a library of *Theileria parva* genes is by the technique of 'SIB selection'. The limiting factor in this process is the extent to which a 'positive' plasmid can be diluted with 'negative' sibs and yet still be detected in a CTL assay. This factor will determine the size of the pools which may be used and, consequently, the number of assays which must be performed.

In order to estimate the pool size feasible for a microcytotoxicity assay, we used a model system consisting of an alloreactive (anti-KN104) CTL line, an expression plasmid containing the KN104 gene (pCD-KN104) and a plasmid expressing an irrelevant gene as background library (pCD-CD8). With this model, we could easily detect specific label release in a COS cell population transfected with 100 ng pCD-KN104

plus 10 ug pCD-CD8 (i.e. 1 positive per 100 background), and clearly, but less extensively, with 10 ng pCD-KN104 per 10 ug pCD-CD8 (i.e. 1/1000). If similar conditions apply for *Theileria parva* antigens reacting with immune CTL, then analysis of a schizont cDNA gene library by SIB selection and microcytotoxicity assays appears feasible.

TH2.4 Attempts to isolate the gene encoding the polymorphic immunodominant molecule of *Theileria parva*, using eukaryotic expression cloning

Scientists: P. Toye, V. Nene, H. Clevers*, P. Wijngaard*, M. Metzelaar*
Research Associate: R. Skilton
Technician: J. Nyanjui

*Department of Clinical Immunology, Academic Hospital, Utrecht, Holland

A cDNA library made from a bovine lymphoblastoid cell line infected with *Theileria parva* (Muguga) was inserted into the COS cell expression vector pCDM8. The quality of this library was demonstrated by the presence in the library of a plasmid containing the gene for the lymphocyte surface antigen, WC-1, which is about 5.5 kbp in size.

After transfection of the library into COS cells, the cells were screened with a panel of monoclonal antibodies which recognize the polymorphic immunodominant molecule expressed in sporozoites and schizonts of *Theileria parva*. This molecule is of interest due to its potential to induce anti-sporozoite immunity (the monoclonal antibodies inhibit sporozoite invasion *in vitro)*, the necessity to determine its role in anti-schizont immunity, and as a potential diagnostic reagent. Cells were identified which reacted with two of the monoclonal antibodies, IL-S36.5 and K5.17.2.2 (MAb 12). The plasmid containing the gene encoding this antigen has now been isolated. Sequence analysis of the 5′ end of the gene indicates that the gene may be incomplete: there are 86 bp upstream of the consensus ATG start site which constitute an open reading frame. In immunological analyses using monoclonal antibodies and immune sera, positive reactions have been observed with only MAbs IL-S36.5 and MAb 12 reacted with molecules of Mr ~ 100 kDa in Western blots of transfected COS cells. Current priority is being given to sequencing the present plasmid, and to isolating a full length copy of this gene.

TH3.1 Immunization of cattle with a recombinant p67 surface antigen of *Theileria parva* sporozoites

Scientists: A. Musoke, V. Nene, S. Morzaria, D. McKeever
Research Associate: C. Nkonge
Technicians: E. Awino, J. Ngugi, L. Gichuru, D. Lugo

The cloning of the gene that encodes p67, the 67 kDa surface protein of *Theileria parva* sporozoites and its expression in *Escherichia coli* have been described in a previous report. The recombinant p67 sequences are fused to the first 85 amino acid residues derived from a non-structural gene (NS1) of influenza virus A. This product (NS1-p67) has been tested in a number of cattle for its capacity to protect against challenge with the parasite.

Immunization with partially purified NS1-p67 emulsified in 3% saponin provided protection in 13 of 21 cattle on homologous challenge with *T. parva* sporozoites. Furthermore 6 of 11 cattle immunized with the antigen were protected against a heterologous stock of the parasite. The latter observation provides evidence that the capacity of p67-specific monoclonal antibodies to cross-neutralize among stocks of *T. parva* is reflected in the ability of cattle immunized with the antigen to resist challenge with heterologous stocks. The factors responsible for the variation that was observed among immunized cattle in their responses to challenge infection are not clear. It seems likely that those animals that showed no response to challenge were able to neutralize the sporozoite challenge sufficiently to prevent establishment of the parasite, whereas mild and severe reactors failed to effect a similar level of neutralization. Antigen-specific serum antibody levels were similar in all immunized animals both in quantity and in neutralizing capacity, and therefore do not appear to correlate with protection. For reasons that are not clear, p67-specific T-cell activity was not detectable in immunized animals up to the time of challenge. It is therefore difficult to speculate as to the exact nature of the protective mechanism that was operative in these animals. Studies are under way to determine the reasons behind the failure to detect T-cell responses in spite of extremely high, isotype-switched, specific antibody responses.

TH3.2 Evaluation of pox virus-based antigen delivery systems for the generation in cattle of protective immune responses against *Theileria* sporozoite antigens

Scientists: M. Juarrero, D. McKeever, M. Mackett*
Technicians: E. Awino, D. Ngugi, W. Mwangi

*Paterson Laboratories, Manchester, UK

Previous reports have described a recombinant vaccinia virus which gives rise to the expression in infected cells of the p67 surface antigen of *T. parva* sporozoites. This recombinant is based on the WR strain of vaccinia and causes the generation of p67-specific antibody and $CD4^+$ T-cell responses. Responses are however poor and are not protective. We have analysed the effect of altering the pathogenicity of the parent vac-

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cinia virus strain on the efficacy of the recombinants as immunizing agents. Additional recombinants have been constructed based on the Wyeth (reduced pathogenicity) and Copenhagen (increased pathogenicity) strains, and these viruses have been assessed in immunization experiments for the capacity to generate p67-specific antibody and cell mediated responses. No differences were evident in the responses of calves to infection with these recombinants. Available evidence suggests that p67 is not expressed on the surface of recombinant virus-infected cells. It is possible that this is related to the observed poor antibody and T-cell proliferation responses in immunized animals. In the coming year it is planned to tailor the p67 gene within the shuttle plasmid so that it is expressed on the surface of infected cells. Recombinant viruses derived from these modified constructs will then be evaluated in immunization trials.

In a separate experiment, a vaccinia virus-based shuttle plasmid has been used to generate a recombinant capripox (CPX) virus that incorporates the p67 gene (CPX-p67). This was accomplished by the use of positive selection in the presence of mycophenolic acid, resistance to which is conferred on the recombinants by the *E. coli gpt* gene, a component of the shuttle plasmid. It is assumed, but has not been established, that the gene has inserted by heterologous recombination in the thymidine kinase (TK) locus of the parent CPX (Kabete Lumpy Skin Disease Virus vaccine strain). The CPX-p67 gives rise to the expression in infected cells of a product recognized by p67-specific monoclonal antibodies, although the size of the major product differs from that of p67 expressed by vaccinia virus or baculovirus. Further characterization of this construct and an evaluation of its immunizing potential in calves are under way.

TH3.3 Analysis of the clonality of bovine CTL responses against *T. parva*

Scientists: D. McKeever, R. Bishop, A. Bensaid*
Technician: A. Kaushal

*Institut d'Elevage et Médicine Vétérinaire Tropicaux (IEMVT), Paris, France

It has recently been possible at ILRAD to clone and sequence α and β T-cell receptor (TCR) genes from cDNA of bovine peripheral blood mononuclear cells and cytotoxic T-lymphocyte (CTL) clones. This has highlighted the possibility of defining the specificity of bovine CTL clones on the basis of the sequences of their rearranged TCR genes. A series of CTL clones has been generated from an immune animal, without *in vitro* restimulation, through the use of lymphatic cannulation techniques. The opportunity therefore exists to assess the extent of the T-cell repertoire that is deployed by an immune lymph node in response to challenge with *T. parva*. In addition, oligonucleotide probes based on TCR sequences may be useful in the detection of parasite-specific CTL before an animal's exposure to the parasite, and so assist in the study of primary CTL responses to the parasite. Using a novel technique based on the polymerase chain reaction, the gene encoding a TCR α chain variable region (Vα) has been cloned from bovine lymphocytes and sequenced. Primers were based on sequences obtained previously at ILRAD and other laboratories. Similar primers have

been designed for the amplification of Vβ sequences. These reagents will be used to clone and sequence Vα and Vβ genes from CTL clones derived from a responding lymph. These experiments will yield information on the extent of the CTL repertoire deployed by an immune animal in response to *T. parva* infection.

TH3.4 Evaluation of novel non-recombinant antigen delivery systems in cattle

Student: A. Diate
Supervisors: D. McKeever, A.J. Musoke, B. Ferry*

*Brunel University, Middlesex, UK

The availability for immunization trials of a recombinant form of p67, the major surface antigen of *T. parva* sporozoites, has highlighted the requirement for efficient antigen delivery systems for cattle. A recent study suggests that immunization with recombinant p67 formulated in saponin can generate high titres of antibody, and protect cattle against challenge with *T. parva*. Titres do not appear to be maintained, however, and the need for an alternative delivery system is clear. The evaluation of available recombinant antigen delivery systems for this purpose is continuing. Several novel non-recombinant systems have been reported recently that give rise to the generation of high titres of antibody in laboratory animals. These have yet to be evaluated for efficiency and acceptability in cattle, but may prove ideal adjuvant formulations for a p67-based vaccine. A controlled trial has been initiated in Boran cattle for the evaluation of three such systems: the Ribi adjuvant system (RAS), Titremax pluronic polyol formulation and immunostimulating complexes (ISCOMS). The responses of groups of five calves to a trypanosomal variable surface glycoprotein formulated in each adjuvant are being measured, with both antibody responses and those mediated by T-lymphocytes being evaluated. It is envisaged that these experiments will provide useful information for the selection of novel adjuvant systems for use in cattle.

TH3.5 Mapping of T-cell epitopes on a *Trypanosoma brucei* variable surface glycoprotein

Scientists: D. McKeever, V. Nene, W.I. Morrison*
Technician: E. Awino

*Institute of Animal Health, Agricultural and Food Research Council (AFRC), Compton, UK

Previous reports have described the characterization of bovine T-cell responses to the variable surface glycoprotein (VSG) of *T. brucei* clone ILTat 1.3, and the generation of $CD4^+$ T-cell clones specific for that antigen. The epitopes of several of these clones have been resolved to three areas on the molecule by the use of deletion products of the gene that encodes the antigen expressed in *E. coli*. On the basis of sequence derived from epitope-defining deletions, two peptides (VSGp254-267 and VSGp364-

384) were designed and synthesized. These peptides were tested for the capacity to stimulate VSG-specific clones and peripheral blood mononuclear cells (PBM) from immune animals. In spite of representing sequence differences between stimulatory and non-stimulatory deletions, neither of these peptides was capable of eliciting responses in the clones or PBM. An additional peptide was therefore synthesized (VSGp247-260), 7 residues of which overlap with one of the original peptides. When tested on the clones that defined the epitope area, VSGp247-260 also failed to stimulate. However, when tested on PBM from VSG-immune cattle, the peptide elicited strong proliferative responses from some but not all animals. The relevance of this observation to the effect of MHC phenotype on the responses of cattle to soluble antigens is discussed in a separate report (see abstract TH3.8). It is concluded that the VSG segment from residues 247-267 contains an epitope for bovine T cells, the recognition of which is dependent on the MHC phenotype of the responding T cells. In the context of the MHC class II restricting element of the clones which defined the epitope, residues present in VSGp254-267 but not VSGp247-260 are crucial for recognition. This may be due to the capacity of these peptides to bind to the MHC molecule.

TH3.6 Identification of a monoclonal antibody that recognizes bovine CD3

Scientists: N. MacHugh, W.C. Davis*

*Washington State University, Pullman, WA, USA

Precise studies of bovine cellular immune responses are highly dependent on the definition of functional subsets with monoclonal antibodies (MAb) that recognize leukocyte surface antigens. A MAb (MM1A) has been identified that reacts with the majority of $CD2^+$ bovine T-lymphocytes and with all $\gamma\delta$ T cells. It does not react with bovine B cells or monocytes. The tissue distribution of MM1A and its reactivity with $CD3^+$ and $CD3^-$ T-cell clones (as defined by Northern blot analysis) are consistent with its recognizing the T-cell receptor-associated CD3 specificity. Immunoprecipitation analysis of ^{125}I-labelled bovine peripheral blood monocytes (PBM) lysates followed by reducing SDS-PAGE revealed a series of bands of approximately 12, 16, 21, 22, 32, 36 and 44 kDa. Under non-reducing conditions an additional band was present at approximately 96 kDa. The true specificity of MAb MM1A was determined by denaturing ^{125}I-labelled PBM lysates before immunoprecipitation with the antibody. Under these conditions MM1A precipitated a dimmer of 21 and 22 kDa. These data suggest that MM1A is reacting with the $\alpha\beta$ or ξ chain of bovine CD3. The additional bands are likely to be the components of both $\alpha\beta$ and $\gamma\delta$ T-cell receptors. Since CD3 is one of the definitive markers of functional T-lymphocytes, MM1A is a valuable addition to the panel of MAbs available at ILRAD.

TH3.7 The biochemical nature and cellular distribution of the bovine γδ T-cell surface antigen WC1

Scientists: N. MacHugh, H. Clevers*, P. Wijngaard*

*Department of Clinical Immunology, Academic Hospital, Utrecht, Holland

The monoclonal antibody (MAb) IL-A29 recognizes the WC1 specificity, a 215 kDa glycoprotein on the surface of bovine γδ T cells. The function of WC1 has not been determined, but cDNA molecules that encode this specificity have recently been cloned from a COS cell expression library. Two of these cDNAs differ significantly at both 3′ and 5′ ends and we have analysed their expressed products in L cells using WC1-specific MAb. Although both L-cell transfectants (WC1.1, WC1.2) stain with IL-A29, each reacts with only one of two other WC1-specific MAb, BAG25A and CACTB32A. These two monoclonal identify non-overlapping subsets of γδ T cells in bovine peripheral blood leukocytes (PBL) but both immunoprecipitate the 215 kDa WC1 glycoprotein. The distribution of cells recognized by BAG25A or CACTB32A shows no tissue specificity, suggesting that the WC1 antigens are not 'homing' molecules.

These data indicate that the WC1 antigen is encoded by a multigene family, of which at least two products incorporate the determinant recognized by IL-A29. The extent of diversity within this family has not been determined, but analysis of both transfectants with a larger panel of γδ T-cell-specific MAbs suggest that a small subset of γδ T cells express yet another distinct WC1 gene product. Future studies in this area will evaluate the extent of polymorphism within the WC1 antigen family, and attempt to identify its ligand(s). This information will provide an insight into the function of these molecules and clarify the role played by γδ T cells in immune responses of cattle and other species.

TH3.8 Influence of MHC phenotype on bovine immune responses to soluble antigens

Student: H. Pereira
Supervisors: A. Teale, D. McKeever, A. Musoke, J. Batchelor*

*University of London, UK

As reported previously, there was no apparent influence of the MHC on recognition of the p67 sporozoite surface antigen of *T. parva* in a group of eight immunized animals selected for homozygosity at the MHC locus. A similar situation has now been observed for the responses of these animals to trypanosomal variable surface glycoprotein (VSG). This may be a reflection of the size of these molecules and their probable incorporation of a range of helper T-cell epitopes to which a number of class II MHC molecules bind. The responses of the group of animals to subunit fragments of these molecules have therefore been analysed in an attempt to demonstrate MHC related effects.

A recombinant fusion protein (TB11) that contains sequences of a predicted T- and B-cell epitope within p67 has been assayed with peripheral blood monocytes (PBM) from each animal. Only one animal (F100) consistently responded to this antigen. PBM from animal G277 and animal F188 also responded to TB11 but these responses were not consistent, varying from assay to assay. A synthetic peptide based on the sequence of the T-cell epitope present in TB11 has yielded similar results.

Bovine T-cell epitopes on the VSG molecule have been identified previously using T-cell clones and expressed products of VSG gene deletions. A synthetic peptide (VSGp247-260) spanning one of these epitopes was evaluated for recognition by the group of immune MHC homozygotes. PBM from animal G277 consistently responded to this peptide, while those from two others responded inconsistently. The remaining animals did not recognize the peptide. The inconsistency of responses obtained *in vitro* to peptide fragments of either antigen may be a reflection of the relative frequencies of specific T cells in PBM, and limiting dilution analyses are under way to address this question. In addition, further work is being carried out to determine if the patterns of responses observed in the group of animals to TB11 and peptide VSGp247-260 are truly MHC-related.

TRYPANOSOMIASIS

TR1.1 The ILCA/ILRAD African Trypanotolerant Livestock Network (ATLN)

Scientist: E. Authié

Research in the Network falls into two areas: epidemiology of trypanosomiasis and trypanotolerance.

In 1991, the predictive value of tsetse challenge on trypanosome prevalence was further assessed. Studies on the epidemiology of drug resistance were conducted in Côte d'Ivoire and Ethiopia (see abstract TR1.3). In the latter site, alternative strategies for trypanosomiasis control in a situation of drug resistance are being evaluated. The ELISA antigen detection test developed at ILRAD has been used in N'Dama cattle from the Gabon ATLN site. Using this technique, 40% of cattle with no detectable parasitaemia were found to be infected and previously unrecorded mixed infections were detected.

Research on trypanotolerance is conducted in The Gambia, Côte d'Ivoire, Gabon and Zaire. The contribution of nutrition to the ability of infected N'Dama cattle to control anaemia was confirmed in studies carried out in The Gambia. Data from Gabon and Zaire showed that the reproductive parameters of N'Dama cows can be affected by trypanosome infections and that partial chemoprophylaxis improves their reproductive performance. Significant interactions between trypanosome and strongyle infections resulting in reduced liveweight gain and PCV have been demonstrated in small ruminants in Côte d'Ivoire. The socioeconomics component of the Network involved cost-benefit analyses of village cattle production under trypanosomiasis risk. Definition of criteria of trypanotolerance was refined through the use of trypanosome antigen detection ELISAs. The greater importance of control of anaemia compared with the control of parasite growth, as measured by parasitaemia or antigenaemia, was confirmed. Animals antigenaemic without detectable parasitaemia may have an ability to control the establishment of infection. The productivity of such animals was similar to that of uninfected animals. Heritability values of the different criteria suggest that, in addition to the level of PCV under high challenge, the presence of antigenaemia without patent parasitaemia could serve as a basis for selection of trypanotolerant animals.

TR1.2 The ILCA/ILRAD African Trypanotolerant Livestock Network (ATLN): Tsetse aspects

Scientist: S. Leak

There are two main projects involving the tsetse component of the ATLN; firstly, the determination of the contribution that tsetse challenge evaluation can make in assessing trypanosomiasis disease risk and planning control strategies.

Monthly collection of data on tsetse population parameters continued at five ATLN sites. Analyses of data sets of more than five years data are under way for improving the precision of parameter estimates in predictive models of relationships between tsetse challenge and trypanosome prevalence. In 1991 detailed statistical analyses were completed of the relationships between tsetse challenge and try-

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panosome prevalence in cattle at Ghibe and Tolley sites in Ethiopia, from 1986 to 1989. A regression analysis at Ghibe of monthly trypanosome prevalences on tsetse challenge throughout five years showed trypanosome prevalence to have a higher correlation with tsetse challenge in the previous month (r=0.60) than the present month (r=0.47). Smaller correlations occurred at Tolley and these also showed the same pattern. This delay would be expected biologically due to the pre-patent period following the feeding on an animal of an infected tsetse.

The second main project concerns biological and economic evaluation of tsetse control using deltamethrin impregnated targets at Ghibe in Ethiopia in an attempt to alleviate a significant problem of resistance to trypanocidal drugs. Five hundred black cotton targets have been deployed in a control area of 150 km^2 and are being used in conjunction with odour attractants. The attractants consist of sachets containing a mixture of phenols, and containers of cow urine and acetone. The trial commenced in May 1990 and is continuing until the end of 1992. The trial was disrupted in May 1991 due to political disturbances in the country. In the first 12 months of the trial the relative density of the main vector, *G. pallidipes* fell from a mean of 1.9 flies/trap/day in the preceding 12 months, to 0.4 flies per trap per day. Before the political disturbances the density had fallen to 0.09 flies/trap/day. There were similar reductions in trypanosome prevalence in adult cattle, falling from a mean of 36% during the 12 months preceding control, to a mean of 14%. The rate of relapse to treatment was also reduced.

Tsetse control using a 'pour-on' insecticide ECTOPOR® started at the second site in Ethiopia, Tolley, at the beginning of 1991.

TR1.3 Epidemiology of drug resistance

Scientists: A.S. Peregrine, E. Authié, J. Rowlands*, W. Mulatu†
Research Associate: S. Leak
Technician: S. Kemei

*International Livestock Centre for Africa (ILCA), Nairobi, Kenya
†International Livestock Centre for Africa (ILCA), Addis Ababa, Ethiopia

Field studies are being conducted at sites in Ethiopia and Cote d'Ivoire to determine the constraints to livestock production and the prevalence of drug resistance. The project in Ethiopia has been conducted at Ghibe, a valley in the south-western part of the country. In 1989, analysis of field data suggested a high prevalence of resistance to the only trypanocide used at the project site; diminazene aceturate. Isolates were subsequently collected from cattle at the project and their drug-resistance phenotypes characterized at ILRAD. Since the study demonstrated a high prevalence of resistance to isometamidium, homidium and diminazene, and that this multidrug resistance was expressed at the clonal level, it was concluded that chemotherapy *per se* would not control the problem. A tsetse control programme using targets impregnated with deltamethrin was therefore initiated in April 1990 in an attempt to alleviate the problem. The relative density of the main vector, *Glossina pallidipes*, fell from a mean of 1.9 flies per trap per day in the 12 months prior to introduction of the control pro-

gramme to a mean of 0.4 flies per trap per day in the 12 months after tsetse control. During the first 3 months of 1991 this figure was constant at 0.09 flies per trap per day. The prevalence of *T. congolense*, which accounts for the majority of cattle infections, has fallen from approximately 30% before the tsetse control programme to a mean of approximately 5% in the first quarter of 1991. There has also been a considerable reduction in the rate of relapse following treatment with diminazene aceturate, from approximately 40% to 20% by the end of the first quarter in 1991.

The project in Cote d'Ivoire involved the examination of 600 cattle at each of 4 project sites (Boundiali, Ferkessedougou, Bouake and Marahoue ranch) and the response of trypanosome infections to treatment with 0.5 mg kg^{-1} isometamidium chloride, 1.0 mg kg^{-1} homidium bromide or 3.5 mg kg^{-1} diminazene aceturate. Due to a very low prevalence of trypanosome infections in cattle the project at Bouake has been terminated. Studies in the other 3 sites, to date, have not demonstrated resistance to any of the three trypanocides. Furthermore, 4 trypanosome populations isolated from cattle at Ferkessedougou were examined at ILRAD and shown to be sensitive in cattle to treatment with diminazene aceturate at a dose of 3.5 mg kg^{-1}. Preliminary data would therefore indicate that there is not a serious problem with drug resistance at these sites.

TR1.4 Phenotypic basis of drug resistance

Scientists: A.S. Peregrine, N.B. Murphy, G. Gettinby*
Technician: S. Kemei
Student: M. Mamman
Supervisors: A.S. Peregrine, P. Gardiner, Y.O. Aliu†

*Department of Statistics and Modelling Science, University of Strathclyde, Glasgow, Scotland
†Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria, Nigeria

Thirty goats were randomly divided into 6 groups of 5 animals each; Groups A, B, C, D, E and F. Each goat in Group A was inoculated intravenously (i.v.) with 10^6 trypanosomes of *Trypanosoma congolense* IL 3274, a clone originating from Burkina Faso. After detection of trypanosomes in each of the goats in Group A, blood was collected from each animal, the level of parasitaemia determined and an aliquot containing 1.0×10^5 trypanosomes inoculated intravenously into one goat in each of Groups B, C and D. Immediately following infection, goats in Group B were treated intramuscularly (i.m.) with diminazene aceturate at a dose of 7.0 mg kg^{-1}; 4 of the animals were detected parasitaemic 13–23 days later. The other goat was not detected parasitaemic for the entire 125-day observation period following treatment. Goats in Group C were not treated and served as infectivity controls for Group B; all were detected parasitaemic within 8 days following the challenge. Three days after all the 5 goats in Group D were first detected parasitaemic, each animal was treated i.m. with diminazene aceturate at a dose of 7.0 mg kg^{-1}. Following treatment, all became aparasitaemic within 48 hours. However, infections in all animals relapsed within 9

days of treatment. On day 15 following treatment, blood samples were collected from each of the 5 animals and the level of parasitaemia quantified in each blood sample. For each blood sample, aliquots containing 1.0×10^5 trypanosomes were inoculated i.v. into two goats each; one in Group E and one in Group F. Immediately after each goat in Group E was infected it was treated i.m. with diminazene aceturate at a dose of 7.0 mg kg^{-1}. The animals in Group F were not treated and functioned as infectivity controls for the animals in Group E. None of the goats in Group E were detected parasitaemic during the 122-day observation period following treatment. In contrast, all animals in Group F were detected parasitaemic, 3–9 days after the challenge. It would therefore appear that when trypanosomes reappear in goats following treatment with diminazene aceturate at a dose of 7.0 mg kg^{-1}, the number of trypanosomes in the population that are resistant to this drug dosage is less than $1:5 \times 10^5$. The results from Group B would indicate that this frequency was much higher prior to drug exposure.

This conclusion was investigated in normal mice which were infected intraperitoneally (i.p.) with IL 3274. When detected parasitaemic the mice were treated i.p. with diminazene aceturate at a dose of 25.0 mg kg^{-1}. After a period of aparasitaemia, infections in all mice relapsed. The blood from these animals was collected, pooled and the level of parasitaemia quantified. The blood was then diluted in phosphate saline glucose and 0.2 ml aliquots containing 10^2, 10^3, 10^4, 10^5 or 10^6 trypanosomes inoculated i.v. into 100 mice each (i.e. 5 groups of 100 mice). Immediately after inoculation, 50 mice in each group were treated i.p. with diminazene aceturate at a dose of 25.0 mg kg^{-1}. The remaining 50 mice in each group were left untreated as infectivity controls. All mice were observed for 100 days following challenge. All infectivity controls became parasitaemic. In contrast, of the mice inoculated with 10^2, 10^3, 10^4, 10^5 or 10^6 trypanosomes and treated with diminazene aceturate, 4/50, 11/50, 13/50, 28/50 and 39/50, respectively, were detected parasitaemic. Analysis of these data indicated that at an inoculum of 10^2, approximately 0.08% of the population were resistant to the drug dosage used. Furthermore, as the size of the inoculum increased the percentage of the population resistant to the drug dosage decreased. Thus, with an inoculum of 10^6 trypanosomes, 0.00015% of the population were resistant. It would therefore appear that the level of resistance expressed by a trypanosome population *in vivo* is dependent on the population size and that most trypanosomes appearing in an animal following treatment are sensitive to the drug dosage that was used.

TR1.5 Development of drug assays for detection and quantification of trypanocides in biological samples

Scientists: A.S. Peregrine, P.H. Holmes*
Research Associate: J. Katende
Student: M. Eisler*
Technician: E. Gault*
Supervisor: P.H. Holmes*

*Department of Veterinary Physiology, University of Glasgow Veterinary School, Glasgow, Scotland

Balb/c mice were immunized with an isometamidium-human serum albumin conjugate (ISMM-HSA) suspended in Complete Freund's Adjuvant. During the following 7 months they were boosted on five occasions with ISMM-HSA suspended in Incomplete Freund's Adjuvant. Splenic cells were then collected from one mouse, fused with a mouse myeloma cell line, X63, and seeded into the wells of eight 24-well tissue culture plates. Ten days later, supernatants from all 192 wells were screened for the presence of antibodies to isometamidium (ISMM) and to human serum albumin (HSA), using a direct ELISA in which plates were coated with either ISMM-HSA or HSA; 17 wells contained antibody which bound to ISMM-HSA and not to HSA. Cells from 16 of these wells were cloned and a total of 222 clones produced. Supernatants from 145 of these wells contained antibody which bound to ISMM-HSA but did not bind to HSA. Eighty-two of these clones were classified as good secretors and the antibody isotypes in these supernatants determined both by ELISA and by immunodiffusion; 6 were IgG_{2b}, the rest were all IgG_1. Studies were conducted with 21 of these clones (including the 6 IgG_{2b} clones) to determine if the antibody they secreted (i) trapped free ISMM and (ii) cross-reacted with homidium or diminazene. An inhibition ELISA was used in which culture supernatants containing 1.0 mg protein ml^{-1} were diluted 1:100 and incubated overnight in solutions containing 10^{-5}–10^{-10}M isometamidium chloride, homidium chloride or diminazene aceturate. The ability of the antibody in these solutions to bind to ISMM was then evaluated with a direct ELISA in which the plates were coated with ISMM-HSA. The binding activity of seven of the antibodies was not inhibited by any of the three trypanocides at concentrations up to 10^{-5}M. The binding activity of 9 antibodies was not inhibited by diminazene at concentrations up to 10^{-5}M but was inhibited by both isometamidium chloride and homidium chloride at concentrations down to 10^{-7}M. Finally, 5 antibodies were not inhibited by either homidium chloride or diminazene aceturate at concentrations up to 10^{-5}M but were inhibited by isometamidium chloride; 3 at concentrations down to 10^{-7}M, and 2 at concentrations down to 10^{-8}M. Therefore, of the 21 antibodies, 7 recognize a conformational epitope involving both ISMM and HSA, 9 recognize ISMM and homidium but do not cross-react with diminazene, and 5 recognize ISMM but do not cross-react with either homidium or diminazene. Three of the 5 isometamidium-specific clones have been recloned and ascites produced.

A competition ELISA for ISMM has been described by others and shown to be a sensitive method for determining the concentration of isometamidium in bovine serum. However, the assay included certain features which were undesirable for use in

field laboratories: very small volumes of test samples were used (5 ul), which may lead to considerable inaccuracies; the second antibody (donkey anti-sheep IgG) used to detect the primary polyclonal reagent (sheep anti-isometamidium IgG) cross-reacted with bovine IgG and could cause confusing background reactions. To overcome these problems the ELISA was modified by pre-dilution of serum samples, and by the use of a biotin-streptavidin system in place of the second antibody. The modified assay was used to determine a statistically acceptable level for the minimum concentration of ISMM that could be detected in serum from East African Zebu cattle. The coefficient of variation (CV) of zero-dose responses was determined for untreated ILRAD Boran *(Bos indicus)* calves (n = 21, CV = 9.4%), untreated ILRAD Boran adult cattle (n = 14, CV = 7.8%) and untreated Boran adult cattle from Kapiti Ranch, Kenya (n = 20, CV = 8.9%). These results were similar to those obtained from Friesian calves in Scotland (n = 24, CV = 9.3%). On the basis of these results it was concluded that the minimum significant concentration of ISMM that could be detected was 50 pg ml^{-1}. In other work, serum samples were collected from Boran cattle 0–86 days after intramuscular treatment with isometamidium chloride at a dose of 0.5 mg kg^{-1} (n = 5) or 1.0 mg kg^{-1} (n = 5) and analysed with the ELISA. The resulting drug profiles showed two distinct phases: an initial rapid decline in concentration (days 1–6) followed by a more gradual decline in concentration with a half-life of approximately 22 days.

TR1.6 Development of *in vitro* drug sensitivity assays

Scientists: R. Kaminsky, E. Zweygarth*, M. Gray*, H. Hirumi, S.K. Moloo
Technician: F. Chuma
Student: P.N. Wasike

*Kenya Trypanosomiasis Research Institute (KETRI), Kikuyu, Kenya

(i) Assay for *T. vivax* bloodstream forms: It was possible to distinguish *in vitro* between two sensitive and two isometamidium-resistant East African *T. vivax* stocks employing a modified version of the long-term viability assay. These experiments have been carried out at KETRI. At ILRAD, we have employed the system for feeder layer-free cultivation of *T. vivax* bloodstream forms and have adapted *T. vivax* stock CP 2331 to axenic growth with growth rates sufficient to carry out 24-hour growth inhibition tests.

(ii) Isolation of trypanosomes with antimicrobials: Many papers contain comments on the use of antibiotics for trypanosome culture systems. Application of antibiotics to the culture media is particularly important for the isolation of trypanosomes from cattle or tsetse to avoid contamination with bacteria or fungi. However, no report exists at present, in which the effect of antimicrobials on bloodstream and insect forms of *T. brucei*, *T. congolense* and *T. vivax* has been quantified (e.g. EC_{50} or long-term viability assay). Employing the 24-hour growth inhibition assay, an anti-trypanosomal effect of Amphotericin B on bloodstream forms of *T. brucei* CP 2469 (clone 1), CP 2137 (clone 1), *T. congolense* CP 81 and *T. vivax* CP 2331 was demonstrated with EC_{50} values of 0.435 µg/ml, 0.153 µg/ml, 0.683 µg/ml

and 0.903 µg/ml, respectively. When incubated for 10 days, *T. congolense* CP 81 could survive 0.75 µg Amphotericin B/ml but not 1.25 µg/ml.

(iii) Direct establishment in culture of trypanosomes from tsetse and from cattle: Isolation of *T. brucei, T. congolense* and *T. vivax* from infected tsetse has been achieved. However, these reports are sporadic and the technique is not a routine procedure. Isolation from cattle has been achieved with *T. brucei* and with *T. vivax*. However, large blood samples are needed to start insect form cultures. Isolation with *T. congolense* has been achieved in insect form cultures. No reports exist on isolation of *T. vivax* or *T. congolense* from cattle into bloodstream form cultures.

(iv) *T. congolense—In vitro* susceptibility of bloodstream forms to diminazene aceturate and isometamidium chloride: The development of a culture technique for the *in vitro* propagation of *T. congolense* bloodstream forms (see abstract TR2.1) made it feasible to employ drug sensitivity assays developed originally for *T. b. brucei*. Five stocks and three clones of *T. congolense* were investigated for their sensitivity to diminazene and isometamidium under axenic culture conditions at 34 °C. Diminazene aceturate produced sigmoidal growth inhibition curves on all *T. congolense* populations. EC_{50} values differed from 0.44 µg drug/ml for IL 1180, a drug-sensitive *T. congolense* clone, to 13.02 µg drug/ml for IL 3338, a drug resistant *T. congolense* stock. EC_{50} values of isometamidium chloride were all in the range of 2.48–9.36 µg/ml. No viable trypanosomes could be detected in any of the *T. congolense* populations after propagation for 10 days in the presence of 100 ng diminazene aceturate/ml, the only exception being IL 3338, which was not effected by the drug at the above-mentioned concentration.

(v) Colorimetric assay for *T. congolense* bloodstream forms: In contrast to *T. brucei* trypanosomes which grow in suspension cultures, most *T. congolense* bloodstream forms grow *in vitro* attached to the surface of the bottom of the culture wells. This unique growth characteristic makes it possible to apply a colorimetric assay protocol (see abstract TR2.1) to *T. congolense* which was originally developed by others for drug screening on cancer cells. Preliminary experiments on *T. congolense* IL 1180, CP 81 and IL 3338 have shown that a modified version of the assay has potential to assess effects of trypanocides on cultured *T. congolense*. When tested with diminazene, the result of the assay was a sigmoidal growth curve for the three *T. congolense* stocks with graphically determined EC_{50} values of 0.04–0.07 µg diminazene aceturate/ml for IL 1180 and CP 81, and 0.6–0.7 µg drug/ml for IL 3338.

TR1.7 *In vitro* studies on mode of action of drugs and drug resistance

Scientists: R. Kaminsky, M. Shaw, A. Peregrine, M. Duszenko*, T. Shapiro†, R. Kratzer§, E. Zweygarth§

*University of Tübingen, Germany
†John Hopkins School of Medicine, Baltimore, USA
§Kenya Trypanosomiasis Research Institute (KETRI), Kikuyu, Kenya

(i) A time-dose response of diminazene on sensitive and drug-resistant *T. brucei* has been established: Bloodstream form trypanosomes of axenically growing populations

were incubated in the presence of 10.0, 1.0 or 0.1 µg diminazene aceturate/ml at 37 °C for various periods and subsequently either inoculated into mice or further propagated *in vitro* in drug-free medium and monitored for their growth for 10 days. The ability of trypanosomes of drug-sensitive CP 2137 (clone 1) to grow *in vitro* was irreversibly damaged after a pulse incubation (<1 minute) with 10.0 µg/ml or after 15 min with 1.0 µg diminazene aceturate/ml. In contrast, drug-resistant CP 2469 (clone 1) trypanosomes tolerated incubation with 10 µg of drug/ml for up to 6 hours and 1.0 µg of drug/ml for up to 24 hours. Differences in drug susceptibility were also detected regarding infectivity to mice and changes in cell volume. The results demonstrated that less than 1 minute exposure to diminazene aceturate at concentrations attained in bovine plasma at the initial peak after diminazene treatment is enough to irreversibly damage drug-sensitive trypanosomes. However, these concentrations were not sufficient to completely eliminate drug-resistant trypanosomes after exposure for 1–6 hours; trypanosomes continued to grow for 48 hours before the majority of them died and only a few organisms survived to revive the cultures.

When drug-sensitive trypanosomes were exposed *in vitro* for 24 hour to diminazene aceturate at the level of concentrations found in cattle after treatment with 3.5 mg/kg, most of the trypanosomes were dead and none of the surviving parasites could be propagated *in vitro* in the absence of drug for more than two days. Simulation of drug concentrations as seen in bovine plasma revealed that only a small population of drug-resistant trypanosomes was not irreversibly damaged. Single surviving trypanosomes were able to establish growing cultures. The addition of feeder layer cells did not change the outcome of these experiments.

(ii) Uptake and efflux of trypanocides by *T. brucei*: The uptake of ^{14}C-labelled trypanocides by two sensitive and two multidrug-resistant *T. b. brucei* clones was followed for 24 hours *in vitro;* no differences were observed in the uptake or efflux of isometamidium chloride between susceptible and drug-resistant trypanosomes. However, uptake of diminazene aceturate was reduced in drug-resistant clones. Resistance in *T. brucei* spp. to diminazene due to reduced drug uptake has been demonstrated previously. These results suggest that multidrug resistance in *T. b. brucei* is not due to one single mechanism but rather due to a combination of individual mechanisms of resistance to each drug.

(iii) Effect of isometamidium on trypanosomes as seen at the EM-level has been demonstrated previously by us. In procyclic forms the kinetoplast appeared particularly to be affected. This does not appear to be the case when bloodstream form trypanosomes are exposed to isometamidium at concentrations equivalent to serum levels in treated cattle.

(iv) S-adenosyl methionine decarboxylase (SAM-DC) inhibition by diminazene and by sinefungin: Preliminary experiments with lysates of drug-sensitive and resistant *T. brucei* revealed that SAM-DC can be inhibited by diminazene. However, the concentrations needed (0.3–1.0mM) are about 30–100 fold higher than drug levels in the sera of treated animals.

TR1.8 Cell biological studies of drug uptake and drug resistance in *T. congolense*

Scientists: A.S. Peregrine, H. Hirumi, D. Zilberstein*
Research Associate: K. Hirumi
Technician: S. Kemei

*Department of Biology, Israel Institute of Technology, Haifa, Israel

The fluorescence property of isometamidium was used to investigate the interaction of the molecule with *in vitro*-derived bloodstream forms of *T. congolense*. Incubation of isometamidium with trypanosomes resulted in a gradual alteration of the lambda max from that of the free compound (600 nm) to that of bound isometamidium (584 nm) in a time-dependent manner. Furthermore, a concomitant increase in the intensity of fluorescence was also observed. Addition of digitonin to trypanosomes caused a rapid increase in fluorescence which was more than four times that observed with intact cells after 180 minutes. This rapid change was observed in cells at 37 °C. It was also observed with cells at 4 °C or when trypanosomes at 37 °C were pretreated with N-ethylmaleimide, where no change in fluorescence was observed prior to the addition of digitonin. These findings indicate that isometamidium has to cross the plasma membrane before its fluorescence is altered. Since N-ethylmaleimide inhibited the changes in fluorescence, the results indicated that transport of isometamidium is mediated via a plasma membrane protein. Furthermore, since this inhibition was immediate and not delayed, it also suggested that trans-membrane transport rather than other internalization processes are responsible for accumulation of isometamidium by *T. congolense*.

TR1.9 Molecular mechanisms of drug resistance in African trypanosomes

Students: R. Jamnadass , G. Ndoutamia
Supervisors: N.B. Murphy, A.S. Peregrine, O.K. ole-MoiYoi

Studies of the molecular basis of drug resistance in trypanosomes have focused on two species, namely *T. brucei* and *T. congolense*. Initial studies were limited to the analysis of a multidrug-resistant *T. brucei* field isolate due to the lack of available, laboratory-derived resistant clones. An interesting observation concerning this isolate was the identification of a 6 kb circular extrachromosomal DNA element in the stock and resistant clones derived from it. Work on this isolate has therefore focused on this element. Presently, both genomic and cDNA clones with homology to repetitive sequences contained on this element have been isolated. Sequence and expression analyses of two cDNA clones of 0.4 kb and 1.4 kb have revealed the following: (i) Both clones contain repeating units of a 113 bp repetitive sequence. (ii) Within the 113 bp repeat lies 2 copies of another 26 bp repeat. (iii) Upstream of the poly A tail of the 0.4 kb cDNA clone is a small microsatellite sequence of 7 CA dimeric repeats. (iv) The repetitive sequence is found predominantly on megabase sized chromosomal DNA molecules in all *T. brucei* isolates analysed. (v) The repetitive sequence is also located on extrachromosomal elements whose copy number would appear to be less than 1 per

genome since these elements are not visible on ethidium bromide-stained gels, whereas haploid chromosomes of similar sizes are. (vi) The repetitive sequence is actively transcribed to generate mature mRNA in all trypanosome isolates examined. (vii) The repetitive sequence has significant homology to a repetitive sequence found associated with Balbiani rings of dipteran species. Sequences from these rings are known to be heavily transcribed in a developmentally-r lated manner.

For *T. congolense*, resistance to quinapyramine sulphate has been induced using a sensitive *T. congolense* IL 1180 clone as the starting clone. This project was initiated following attempts to reintroduce quinapyramine sulphate as a prophylactic against trypanosomiasis in cattle. It has been suspected that this drug can induce cross resistance to other trypanocides, but this has never been clearly demonstrated in the laboratory. Cross resistance studies in mice have demonstrated that a 40 fold increase in quinapyramine sulfate resistance is associated with a 5, 7 and 28 fold cross resistance to diminazene aceturate, isometamidium chloride and homidium chloride respectively. Molecular biology studies to identify the basis of this drug resistance phenotype are currently under way.

TR1.10 Mapping of the *T. congolense* genome

Scientists: N.B. Murphy
Research Associate: E. Gobright.
Technicians: P. Pandit, S. Wasike

All of the *T. congolense* size classes of chromosomal DNA molecules can now apparently be separated by pulsed field gel electrophoresis (PFGE). Genes that are currently under study at ILRAD, and elsewhere, can now be assigned to their respective chromosomes. In addition to this, separated chromosomes can be digested by restriction enzymes and the resulting fragments further separated on another gel. This technique is particularly useful for clusters of chromosomes that cannot be separated by standard PFGE techniques.

Additional markers are currently being generated following the identification of microsatellite sequences in the *T. congolense* genome. One such microsatellite has previously been identified at the trypanothione reductase locus of the *T. congolense* genome and, more recently, another one has been identified at the locus of the p17 cytoskeletal gene. Other microsatellite sequences are currently being analysed following the screening of a *T. congolense* genomic library of sheared DNA fragments of approximately 400 bp in size.

TR1.11 Genetic recombination in *Trypanosoma congolense* isolates from East Africa

Scientists: R.A. Masake, V.M. Nantulya, S.K. Moloo, P.A.O. Majiwa, L.H. Otieno*
Research Associate: S. Minja
Technicians: H. Gathuo, I. Okumu, J.M. Makau, J.T. Njuguna and S. Ngava

*International Centre for Insect Physiology and Ecology (ICIPE), Nairobi, Kenya

To date there are no data, experimental or otherwise, on the occurrence of genetic exchange amongst *Trypanosoma congolense* parasites. The present study, therefore, seeks to address the question as to whether or not genetic exchange occurs naturally in *Trypanosoma congolense* and, if it does, to establish where in the life cycle the exchange takes place, and the frequency of the occurrence. We co-transmitted K45/1 and IL 1180 through tsetse-flies. After cyclical development of the parasites in tsetse, the tsetse were fed on mice. We have analysed the populations arising in the mice and cloned the trypanosomes from those reacting with both Kilifi-type and Savannah-type specific-DNA probes. Twenty-five of the 65 clones obtained so far hybridized only with the DNA probe specific for the savannah-type *T. congolense*.

TR1.12 Attempts to demonstrate genetic recombination between diminazene-resistant and suramin-resistant *T. b. brucei* clones in *G. m. centralis*

Scientists: E. Zweygarth*, R. Kaminsky, N. Murphy, A. Peregrine, S. Moloo
Technicians: J. Kabata, H. Gathuo, A. Adema, F. Chuma, I. Okumu, C. Sabwa, F. Waweru

*Kenya Trypanosomiasis Research Institute (KETRI), Kikuyu, Kenya

Two groups of sublethally irradiated rats were infected with *T. b. brucei* clones IL 3565 or IL 3509. *T. b. brucei* clone IL 3565 was derived from diminazene-resistant stock CP 2469 which was isolated from a Zebu steer in Hakata, Somalia in 1985. *T. b. brucei* clone IL 3509 was derived from suramin-resistant stock CP 1154 which was isolated from a camel in Korbesa, Kenya, in 1982. Confirmation of their drug sensitivities was obtained following cultivation *in vitro*.

Bloodstream form cultures of *T. b. brucei* clones IL 3565 and IL 3509 were initiated from tail blood of infected mice. Both clones did not grow *in vitro* under axenic conditions but required fibroblast-like feeder cells for initiation of cultures. After establishement of these cultures, trypanosomes were adapted to feeder layer-free culture conditions. The two clones were tested *in vitro* for their sensitivity to diminazene and suramin. Graphically determined EC_{50} values in the 24-hour growth inhibition assay were 0.13 µg/ml diminazene aceturate for IL 3509 and 0.85 µg/ml for IL 3566. When incubated for 3 hours in the presence of 1 µg/ml diminazene aceturate and thereafter propagated *in vitro* without drug, all samples of clone IL 3565 continued to grow but all cultures of clone IL 3509 died. Graphically determined EC_{50} values in the 24-hour

growth inhibition assay were 0.83 g/ml suramin for clone IL 3565 and 24 g/ml suramin for clone IL 3509.

Trypanosomes of both clones were taken from rats at the peak of parasitaemia. Heparinized blood was mixed and fed to *G. m. centralis in vitro* in the ratio of 1:1 (P_1/P_2). Further flies were fed either on *T. b. brucei* IL 3565 (P_1) or IL 3509 (P_2). The tsetse were maintained on three different rabbits for 26 days, were left unfed for 2 days and then were probed onto warmed slides at 37 °C to identify those with mature infection. The infection rates were the following: *T. b. brucei* 'hybrid' P_1/P_2, 14 with metacyclics (5.2%); P_1, 1 with metacyclics (0.6%); and P_2, 4 with metacyclics (2.7%). On day 33 after infection, tsetse with a mature infection were fed on mice, and at peak of parasitaemia the infected blood of each mouse was cryopreserved in liquid nitrogen.

Individual mice were infected with tsetse-derived metacyclics of *T. b. brucei* IL 3565 (P_1), IL 3509 (P_2) (control groups) or IL 3565/3509 (P_1/P_2; 'hybrid' group). At peak parasitaemia, groups of mice were infected with 1×10^5 trypanosomes/mouse derived from an individual mouse. The groups were treated i.p. after 24 hours with 50 mg/kg diminazene aceturate or 30 mg/kg suramin. In groups infected with metacyclics derived from tsetse after 36 days of their infective blood-meal, controls relapsed after treatment with diminazene aceturate and suramin, respectively. However, in the 'hybrid' group, relapses occurred only after suramin treatment.

In groups infected with metacyclics derived from tsetse after 39 days of their infective blood-meal, controls relapsed after treatment with diminazene aceturate and suramin, respectively. In the 'hybrid' group, relapses after diminazene aceturate and suramin were observed. Relapse populations were then used to infect new groups of mice, the treatment was performed as above; relapses after diminazene aceturate treatment were retreated with suramin and relapses after suramin treatment were retreated with diminazene aceturate. No relapses occurred following such double treatments.

Another group of mice infected with metacyclics derived from tsetse 88 days after their infective blood-meal was treated with a mixture of diminazene aceturate and suramin 24 hours after the tsetse bites. Relapses did not occur in this instance either.

Thus, if genetic recombination occurred during the co-transmission of the two *T. brucei* clones, it did not lead to the dual expression of the two drug-resistance phenotypes portrayed by the parental clones.

TR1.13 Studies of *T. vivax* from South America

Scientists: M. Dirie, J. Otte*, M. Desquesnes†, P.R. Gardiner,
Technicians: R. Thatthi, A. Adema

*Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ), Calle, Colombia
†Institut d'Elevage et de Médecine Vétérinaire des Pays Tropicaux (IEMVT), Cayenne, French Guyana

In South America *Trypanosoma vivax* is transmitted mechanically by biting flies. This fact and the number of occasions on which *T. vivax* might have been introduced into the sub-continent would both influence the extent of genetic and antigenic heterogeneity expressed by *T. vivax* isolated from South America compared with their African counterparts.

Four *T. vivax* isolates from different localities in Colombia have been studied and compared with African *T. vivax* as follows: stabilates of the Colombian parasites from Lorica, Monteria, Palmira and Dorada all grew well in dairy calves, held as a precaution in isolators for the first 30 days. Further stabilates from each of the four isolates were prepared and sufficient parasites isolated to carry out isoenzyme and DNA probe analyses on lysates, and karyotype analysis (new for *T. vivax)* on one isolate. Each of these tests also included representative parasites from the 'West African group' of *T. vivax* and from the 'Kenyan group'. As expected, the Colombian parasites, although showing minor isoenzymic differences amongst themselves and from the Nigerian parasite we chose to represent the West African group, were clearly allied to the West African group, as others have found, and not with the Kenyan parasites. This grouping was confirmed by DNA probe analysis.

Using live parasites from early calf infections we could not demonstrate the infectivity of these parasites for either mice or tsetse—the parasites existing for less than 24 hours after ingestion by tsetse. Importantly, as judged by cross-lysis tests with 30, 60 and (in two cases) 90 day sera, all the Colombian stocks cross-react extensively, but do not react with a late infection serum which lyses the Nigerian parasites. In these tests however the Monteria parasite was susceptible to lysis by even non-specific sera suggesting that it is especially fragile. As a further corollary of antigenic relatedness, calves infected with the Palmira and Dorada isolates were allowed to cure their infections without treatment—which took over 90 days—and then both calves were challenged with stabilates of the Dorada parasites. Both calves resisted infection whereas a naive calf did not, demonstrating, in support of the cross lysis tests, that the Palmira and Dorada isolates are serologically similar. Further challenge infections suggested that all four Colombian parasites come from a single serodeme.

Work is also in progress to determine the prevalence of *T. vivax* infection rates in livestock in French Guyana and to ascertain the relative importance of this parasite vis-a-vis other haemoparasitic diseases.

TR1.14 The development of DNA probes for use in identification and characterization of trypanosomes

Scientists: P.A.O. Majiwa, O.K. ole-MoiYoi

For each trypanosome species-specific repetitive DNA sequence(s) identified to date, a pair of oligonucleotide primers has been optimized for use in amplification by the polymerase chain reaction. These have been used in combination with (α^{32}P)-dNTP-based labelling systems to reveal the presence of parasites in both livestock and tsetse at levels undetectable by hybridization alone.

These probes have been employed in the analysis of samples collected in the field from both tsetse flies and infected animals. Protocols for sample processing and storage have been developed and have enabled us to test samples several months after their collection. In such samples signals were still detectable, albeit at lower intensities, in all those which tested positive a few days after collection.

TR1.15 Development of improved techniques for diagnosis of African trypanosomiasis

Scientists: R.A. Masake, V.M. Nantulya, O.K. ole-MoiYoi
Research Associate: S. Minja
Technicians: J.M. Makau, J.T. Njuguna, H.Gathuo, S. Ngava

Monoclonal-based antigen-ELISAs developed by ILRAD scientists have now been tried out in the field and found to be above 95% sensitive in the identification of *Trypanosoma vivax, Trypanosoma brucei* and *Trypanosoma congolense*. The antigens recognized by the 3 species-specific monoclonal antibodies are currently being worked on. The *T. vivax* species-specific diagnostic antigen has been identified in all the *T. vivax* parasites tested on Western blots, which includes *T. vivax* parasites from Kenya, Uganda and West Africa. In freshly prepared *T. vivax* lysates, the antigen varies in size ranging from 8–10 kDa to more than 100 kDa. Following storage at –20 °C and subsequently thawing, the antigen tends to break down to the 8–10 kDa product. This band has been electro-eluted from SDS-PAGE gels, inoculated into rabbits and monospecific polyclonal antibody capable of recognizing multiple bands on Western blots of *T. vivax* lysates obtained. The resultant monospecific polyclonal antibody was applied to the identification of the *T. vivax* species-specific antigen in a cDNA library. The cloned gene was expressed in pUVR291 in xL-1 cells to produce fusion protein, MARA I. MARA-1 has been tested in ELISA system found to react both indirectly and in antigen-capture ELISA. Preliminary electron microscopical studies show that the *T. vivax* diagnostic antigen is intracellular and diffusely distributed throughout the cytoplasm.

The study of *T. brucei* monoclonal antibodies has led to identification of 4 species-specific monoclonal antibodies. Three of these antibodies react with multiple protein bands on Western blots while one of them detects only one band of Mr 50 kDa. This protein band is currently being electroeluted and used for immunizing rabbits to facilitate production of monospecific antisera. The resultant sera will be

used in identification of the gene encoding this *T. brucei* species-specific antigen in λgt11.2 expression cDNA library.

TR1.16 Development of monoclonal antibody-based assays for species identification of trypanosomes in the tsetse fly *(Glossina spp.)*

Student: K.M. Bosompem
Supervisors: V.M. Nantulya, R.A. Masake, S.K. Moloo, R.K.G. Assoku*

*Department of Animal Science, University of Ghana, Accra, Ghana

Several trypanosome species- and subspecies-specific monoclonal antibodies (MAbs) have been produced against procyclics of *T. congolense, T. simiae* and *T. brucei* and epimastigotes of East and West African *T. vivax*. The MAbs were obtained following immunization of mice with crude extracts of trypanosomes and selected by immunofluorescence assays and ELISA.

The MAbs have been utilized to develop a sensitive and specific nitrocellulose (NC) based dot-enzyme immunoassay for differentiating *in vitro*-propagated insect forms of *T. congolense, T. brucei* and *T. vivax* and the *Nannomonas* subspecies *T. simiae* and *T. congolense*. Dot-applied trypanosome suspensions on NC were probed with unlabelled specific MAb and revealed by enzyme labelled anti-mouse IgG. Evaluation of the sensitivity of the MAbs in the dot-enzyme immunoassay indicated that some of the *T. brucei, T. congolense* and *Nannomonas*-specific MAbs could detect as low as 10 organisms per dot, whilst the *T. vivax* MAbs detected a minimum of 1,000 organisms per dot. The assay had a specificity of 100%. Although no MAb specific to *T. simiae* has yet been produced, the presence of *T. congolense* and *Nannomonas*-specific MAbs made it possible to identify *T. simiae* by exclusion.

The specificity of some of the MAbs has been confirmed by cross reactivity analysis using the more sensitive Western blot analysis. Of the four *T. brucei* specific MAbs, TR7/47.37.16, an IgM, recognized a triplet of protein bands between 30–38 kDa whilst KT43/33.32, an IgG_1, recognized multiple bands between 30–70 kDa with bigger bands towards the higher molecular weight. The third *T. brucei* antibody KT39a/18.17, an IgM, recognized a 90 kDa protein band whilst the fourth, KT43/27.32, an IgG_{2a}, did not work in this assay. The *T. vivax*-specific antibody KT32/48.16.34, an IgG_1, produced following immunization with *T. vivax* epimastigote antigens, did not recognize any bands using *T. vivax* bloodstream form lysates. Also, this MAb did not react with whole bloodstream form *T. vivax* trypanosomes in the 'live immunofluorescence' assay, even though it reacted with epimastigotes by the same method, thus suggesting stage specificity. Western blotting analysis using epimastigote antigens is yet to be done. C2 and TC6/42.6.3, both *T. congolense*-specific and of IgG_1 isotype, recognize protein bands within the same molecular weight range (30–40 kDa). C2 binds a doublet of protein bands at 30 and 31 kDa and another doublet at 38 and 40 kDa, whilst TC6/42.6.3, recognizes only one band at 31 kDa.

After periodate oxidation treatment, ELISA demonstrated that two out of four *Nannomonas*-specific MAbs (KN4/13.9(IgG_3) and KN5/6.15(IgG_1)) detected periodate-sensitive, carbohydrate antigenic determinants in glycoprotein or glycolipid anti-

gens, whilst all the *T. congolense, T. brucei* and *T. vivax* specific MAbs detected non-carbohydrate or periodate-insensitive carbohydrate antigenic determinants.

Preliminary studies on the application of the Dot-ELISA to the detection of experimentally infected *Glossina* indicate that 80% of *T. congolense* IL 1180 infected male *Glossina morsitans centralis* flies could be detected reliably.

TR1.17 Identification, cloning and characterization of the gene encoding the diagnostic antigen for *Trypanosoma congolense* infections.

Student: A. Jaye
Supervisors: P.A.O. Majiwa, V.M. Nantulya, O.K. ole-MoiYoi, K.M. Hudson*

*Brunel University, Middlesex, UK

The species-specific diagnostic monoclonal antibody (TC39, an IgM isotype) recognizes multiple bands of proteins (Mr of between 38 and 50) in Western blots using freshly prepared *T. congolense* lysates. The electroeluates of these bands have been used to raise IgG class monoclonal antibodies. One of the monoclonal antibodies produced, C2 (an IgG_1 isotype), reacts strongly with *T. congolense* lysates by Western blotting. The monoclonal antibody recognized the antibody 38 and 40 kDa, as well as a doublet of 30 and 31 kDa. The antibody was specific for *T. congolense*, and did not react with lysates from either *T. vivax* or *T. brucei* in Western blots or sandwich ELISA assays. The monoclonal antibody C2 has been compared with TC39 in the sandwich antigen capture ELISA using both *T. congolense* lysates and infection sera. The pattern of capture of the antigen was similar; and one conjugated antibody also bound to the antigen captured by the other. Thus, the epitopes recognized by both monoclonal antibodies may be present on the same antigen molecule. Chemical analysis by periodate oxidation and proteinase-K digestion of lysates indicated that the antigen detected by both monoclonal antibodies is a protein.

Rabbits were immunized with the 38/40 kDa protein eluates recognized by C2 to produce polyvalent antisera (R-AT9 and R-BT9). The polyvalent sera was used for screening a *T. congolense* cDNA library in the expression vector λgtII. Nine clones were identified that reacted with the antiserum.

TR1.18 Analysis of *Trypanosoma congolense* stocks by molecular karyotyping

Student: Rebecca Waihenya
Supervisors: Dr. R.A. Masake, G.K. Kinoti*

*Department of Zoology, University of Nairobi, Kenya

Trypanosoma congolense isolates from East Africa (Kenya and Uganda) and West Africa (Gambia, Burkina Faso and Nigeria) were grown and cloned in mice. The DNA of the derived clones was subjected to Orthogonal Field Alternating Gel

Electrophoresis (OFAGE) in order to investigate the genomic relationships amongst the clones.

All the clones studied displayed one overall molecular karyotype characteristic of *T. congolense* IL 1180 (Savannah type). The overall pattern was characterized by the presence of relatively small mini-chromosomes (approx. 50–150 kb) and lack of medium-sized chromosomes in the range of 200–400 kb. Furthermore there was no clear cut distinction between clones from eastern and western regions of Africa. Similarities in chromosome profiles were frequently observed between clones from the same stock while distinct variations were noted in the chromosome-sized DNA molecules of clones belonging to different trypanosome isolates. A few clones derived from different trypanosome stocks did however exhibit similar chromosome patterns.

Cross protection experiments carried out in mice demonstrated that clones exhibiting similar chromosome profiles offered protection against each other while those with different profiles did not. The chromosome profiles of several clones remained stable following chronic infection in goats and transmission through tsetse flies.

TR1.19 Maintenance of production colonies of different *Glossina* species

Scientist: S.K. Moloo
Technician: N. Kuria

The Tsetse Vector Unit maintains breeding colonies of tsetse flies. Trypanosome parasites normally pass through several stages of their life cycle in the tsetse fly. Although it is possible to maintain trypanosomes *in vitro* and to infect laboratory animals and livestock by injecting parasites into the animals, trypanosomes maintained and transmitted artificially differ from parasites transmitted naturally through the bite of an infected tsetse fly. For this reason, a great deal of the trypanosomiasis research conducted at ILRAD requires trypanosomes that have developed in tsetse flies. Thus, researchers studying trypanosomiasis collaborate closely with staff of the Tsetse Unit.

Five tsetse breeding colonies were maintained at ILRAD in 1991. These were *Glossina morsitans centralis* which originated from mainland Tanzania; *G. pallidipes* from Kenya; *G. palpalis gambiensis* from Burkina Faso; *G. brevipalpis* and *G. longipennis*, both from Kenya. These species represent the three taxonomic groups of tsetse—*morsitans, palpalis* and *fusca*. All breeding colonies were maintained at 25 °C and were fed 5 days a week on the ears of half-lop rabbits. *G. m. centralis* and *G. longipennis* were kept at 70% relative humidity, and the others at 80%. The mean numbers of breeding females of *G. m. centralis, G. pallidipes, G. gambiensis, G. brevipalpis* and *G. longipennis* colonies were 10273, 2945, 1134, 1573, 2162; they produced 304227 (mean wt. 34.7 mg), 67924 (41.3 mg), 38305 (29.9 mg) 42868 (77.7 mg) and 50249 (77.5 mg) puparia, respectively. These colonies provided all the tsetse required for trypanosomiasis research conducted at ILRAD in 1991, and also provided research groups in Kenya and abroad with tsetse puparia.

TR1.20 Vector competence of different *Glossina* species for pathogenic trypanosomes

Scientist: S.K. Moloo
Technicians: J.M. Kabata, C.L. Sabwa, N.M. Kuria

A study was undertaken to determine if there were differences in the innate susceptibility of 7 different *Glossina* species and subspecies for *Trypanosoma vivax, T. congolense,* or *T. brucei brucei* stocks isolated in East and West Africa. Teneral *Glossina morsitans centralis* (origin, Tanzania), *G. austeni* (Zanzibar), *G. palpalis palpalis* (Nigeria), *G. p. gambiensis* (Burkina Faso), *G. fuscipes fuscipes* (Central African Republic), *G. tachinoides* (Chad) and *G. brevipalpis* (Kenya) from the laboratory-bred colonies were allowed to feed simultaneously for 24 days on Boran cattle infected with *T. vivax,* for 24 days on goats infected with *T. congolense,* or for 34 days on goats infected with *T. b. brucei* from East or West Africa. The tsetse were then dissected. Infection rates were as follows: 0–97% for *T. vivax;* 0.3–49.2% for *T. congolense* and 0–40% for *T. brucei.* Cyclical development of *T. vivax* from both Likoni and Galana, Kenya, was best in *G. m. centralis* (61.1%, 36.2%) and in *G. brevipalpis* (75.3%, 58.2%) but poor in *G. austeni* (1.8%, 5.0%) and the four *palpalis* group tsetse (range, 0–4.9%). For the stock from Bamburi, Kenya, the infection rates were high in all seven tsetse species used (range, 15.3–91.3%). For the two Nigerian *T. vivax* stocks, the infection rates were also high in all the seven tsetse species, which showed a range of 55.5% to 97.1%. Cyclical development of both the Tanzanian and the Nigerian *T. congolense* was best in *G. m. centralis* (35.3%, 49.2%), poorest in *G. austeni* (2.0%, 3.0%) and the four *palpalis* group tsetse (range, 0.3–6.0%), with *G. brevipalpalis* intermediate (15.7%, 6.3%). The infection rates of both the Tanzanian and the Nigerian *T. b. brucei* were high in *G. m. centralis* (40.4%, 6.8%), but low in the other tsetse species (range, 0–2.1%). Thus, there were differences in the vector competence of different *Glossina* species and subspecies for the above *T. vivax, T. congolense* and *T. brucei* populations.

TR1.21 Susceptibility of *G. pallidipes* and *G. m. centralis* to pathogenic *Trypanosoma* species

Scientist: S.K. Moloo
Technicians: C.L. Sabwa, J.M. Kabata

Susceptibility of teneral *Glossina pallidipes* to pathogenic *Trypanosoma* species was compared to that of *G. morsitans centralis.* Cattle or goats were used as reservoir hosts to feed teneral tsetse, rabbits were used to maintain tsetse which were dissected on day 30. Mean infection rates of *G. pallidipes* and *G. m. centralis* by *T. vivax* isolated from a cow in Kenya were respectively 39.5 ± 8.9% and 32.1 ± 10.3% whilst for *T. vivax* isolated from a cow in Nigeria, they were 30.0 ± 7.5% and 19.8 ± 4.3%. The differences were not significant. Differences between the sexes were also not significant. The transmission rates of the two *T. vivax* isolates from goats in either tsetse species were high. Mean infection rates for *T. congolense* isolated from goats by

either tsetse species were high. Mean infection rates by *T. congolense* isolated from a lion in Tanzania were significantly lower in *G. pallidipes* (8.5 ± 1.8%) than in *G. m. centralis* (22.5 ± 2.0%). Males of either tsetse species were significantly more susceptible than females. Transmission rates to goats and mice by both tsetse species were high. *G. pallidipes* (3.5%) was less susceptible than *G. m. centralis* (25.1%) to *T. congolense* isolated from a cow in Nigeria, but transmission rates in goats and mice by either tsetse were high. Also, *G. pallidipes* (2.7 ± 0.4%) was significantly less susceptible than *G. m. centralis* (18.4 ± 1.1%) to *T. b. brucei* isolated from a hartebeest in Tanzania. Males of either tsetse species were significantly more susceptible than females. Transmission rates in goats and mice by either tsetse were high. *G. pallidipes* (0%) was not susceptible to *T. b. brucei* isolated from a pig in Nigeria whilst *G. m. centralis* showed an infection rate of 9.3%. However, when male *G. pallidipes* and *G. m. centralis* were fed every day for 27 days on a goat infected with this *T. b. brucei* from Nigeria, the infection rates were 8.7% and 20.2%, respectively. The transmission rates in mice by either tsetse species were high. In conclusion, both sexes of *G. pallidipes* show vector competence equal to those of *G. m. centralis* for the two *T. vivax* isolates used, but male *G. pallidipes* and *G. m. centralis* are more susceptible to both *T. congolense* and *T. b. brucei*. Whereas *G. pallidipes* is as susceptible as *G. m. centralis* to *T. vivax* from Kenya and Nigeria, *G. pallidipes* is less susceptible than *G. m. centralis* to *T. congolense* and *T. b. brucei* from Tanzania and Nigeria.

TR1.22 Susceptibility of geographically isolated populations of *Glossina pallidipes* to *Trypanosoma congolense* from Kenya

Scientists: S.K. Moloo
Technicians: I. Okumu, C. Sabwa

Two colonies of *G. pallidipes* were established using pupae which were deposited as larvae by wild female tsetse caught in Shimba Hills, Coast Province, and in Nguruman, Rift Valley Province, Kenya. They were maintained at 25 (±0.5) °C, alternate 12 hour dark/light periods, and at 80% relative humidity, and were fed five days per week on rabbits' ears. Virgin female tsetse were mated when 7 days old to males 10–15 days old. The sexes were left together for 7 days. Female tsetse were kept for 90 days before discarding. Survival and reproductive performance of the two *G. pallidipes* colonies were satisfactory, and both colonies provided adequate surplus to compare their vector competence for *T. congolense* stocks.

Teneral male and female *G. pallidipes* from the colony which originated from Shimba Hills (SH) or Nguruman (NG) were fed simultaneously on 2 goats which were infected with *T. congolense* stocks isolated from *G. pallidipes* in Shimba Hills or Nguruman, Kenya. They were then maintained on rabbits, and on day 25 the surviving tsetse were dissected. Infection rates of five additional groups of teneral tsetse from the two colonies were similarly determined after feeding them on the above two goats on five different days during the course of infection. The mean mature infection rates (X, ± S.E) in SH and NG *G. pallidipes* by *T. congolense* from Shimba Hills were 4.9, ± 1.5%, and 16.4, ± 2.8%, whilst by *T. congolense* from Nguruman, the rates were 5.6, ± 1.4%, and 19.3, ± 3.8%, respectively. The differences were significant. Thus, *G.*

pallidipes from Nguruman is more susceptible than those from Shimba Hills to *T. congolense* stocks isolated from *G. pallidipes* from the two areas of Kenya.

TR1.23 African buffalos, N'Dama and Boran cattle as reservoirs of *Trypanosoma congolense* for *Glossina*

Scientist: S.K. Moloo, R.O. Olubayo*
Technicians: J.M. Kabata, I. Okumu

*Kenya Agricultural Research Institute (KARI), Muguga, Kenya

Teneral *Glossina morsitans centralis* were fed on the flanks of African buffalo, N'Dama or Boran cattle infected with *Trypanosoma congolense*. The infected tsetse were maintained on rabbits and on day 30 after the infected feed, the surviving tsetse were dissected to determine the infection rates. The mean infection rates (±SE) were 23.5 ± 3.3%, 31.6 ± 2.7% and 33.7 ± 4.6%, respectively. The differences were not significant. However, the mean mature infection rate in tsetse fed on the buffalo (13.2 ± 2.1%) was significantly lower compared to the rates in tsetse fed on the N'Dama (20.4 ± 1.4%) or the Boran cattle (21.4 ± 1.1%). When groups of teneral *G. m. centralis*, *G. pallidipes*, *G. p. gambiensis*, *G.f. fuscipes*, *G. brevipalpis* and *G. longipennis* were fed simultaneously on either an infected buffalo, an N'Dama or a Boran steer, the mature infection rates ranged from 0 to 16.1%. Irrespective of the host species used, the *T. congolense* infection rate was highest in *G. m. centralis*, lowest in the *palpalis* and *fusca* group tsetse, with *G. pallidipes* being intermediate.

TR2.1 *In vitro* cultivation of African trypanosomes and their characterization

Scientists: H. Hirumi, Y. Yabu, S.K. Moloo, A.S. Peregrine
Research Associate: K. Hirumi
Technician: J. Wando

Recently, axenic culture systems for cultivating bloodstream forms (BSFs) of *T. brucei* (in 1989) and *T. congolense* (in 1990) have been developed by using newly designed HMI-9 and HMI-93 media, respectively. Using these systems, BSFs of a clone (GUTat 3.1) and a stock (IL 2343) of *T. brucei* and 2 clones (ILNat 3.1 and IL 3000) and 4 stocks (IL 2079, IL 2466, IL 3266 and CP-81) of *T. congolense* were continuously cultivated in 1991. Among these, the axenic cultures of *T. congolense* ILNat 3.1 (a derivative of IL 1180 known to be sensitive to isometamidium) was utilized for an analysis of the interaction of isometamidium with *Trypanosoma congolense.*

An axenic culture system for *T. vivax* BSFs has also been developed by using HMI-163 medium which was modified from HMI-93 medium. Using this system, 'drug-sensitive' (IL 1392) and 'drug-resistant' (IL 3671) clones of *T. vivax* were continuously cultivated for more than 90 days. In addition, BSFs of a clone (IL 2642, known to be highly sensitive to diminazene and isometamidium) and a stock (IL 3338, known to be highly resistant to the drugs) of *T. congolense* were also successfully cultivated by using HMI-165 medium which was further modified from HMI-163 medium.

A simple *in vitro* method for detecting the levels of sensitivity of *T. congolense* and *T. vivax* BSFs to diminazene, isometamidium, homidium and quinapyramine has been developed using the axenic cultures of these 'drug-sensitive' and 'drug-resistant' BSFs. Results obtained to date indicate that levels of drug-sensitivity could be readily detected on 24-well culture plates within 3–5 days by means of phase-contrast microscopy and colorimetry (phenol red). The method may become a useful tool for investigating the sensitivity of the parasites to trypanocidal drugs and for studying the mechanisms involved in drug resistance.

TR2.2 Trypanosome receptors and endocytosis

Scientists: D.J. Grab, J.D. Lonsdale-Eccles, J. Naessens, O.K. ole-MoiYoi, M. Shaw
Research Associates: C. Wells, G. Mpimbaza
Technicians: Y. Verjee, S. Wasike

Transferrin Receptor: Because of the importance of iron-loaded transferrin to trypanosome growth, we have sought to identify the relevant receptor protein(s), as well as to determine whether immunological intervention using anti-transferrin receptor antibodies can alter the parasite's growth. Trypanosomes *(T. brucei)* were labelled with ^{35}S-methionine prior to lysis with Triton X-100. The lysate was subjected to affinity chromatography using diferric transferrin-Sepharose. Gel filtration studies suggest that the molecular mass of the protein fraction eluted from transferrin-Sepharose under acidic conditions was 165 kDa. Incubation of the

transferrin-Sepharose fraction with diferric transferrin yielded higher molecular mass complexes. In other studies, ^{125}I-diferric transferrin was incubated with the detergent lysate prior to gel filtration chromatography. Under these conditions, iodinated transferrin was found to be associated with a 250–300 kDa protein complex. The putative transferrin/receptor complexes could be immunoprecipitated with anti-transferrin antibody. Under reducing conditions, a major protein of 82 kDa, and some minor proteins, were observed on SDS-PAGE. The data suggests that a transferrin receptor-like protein is present in *T. brucei* which is a dimeric molecule capable of binding at least one molecule of transferrin. On Western blots, five different monoclonal antibodies to the human transferrin receptor failed to specifically recognize any trypanosome protein(s). However, preliminary studies suggest that polyclonal antisera (prepared in rats) made against the putative transferrin-receptor protein is capable of killing *T. brucei* organisms in an *in vitro* culture system.

Heterotrimeric G-Proteins: The above example uses a direct approach to identify a particular receptor. Another, and possibly a more fruitful, approach is to study a class of GTP-binding proteins (G-proteins) involved in signal transduction. Heterotrimeric (α, β, γ subunit structure) G-proteins are 85–90 kDa molecules known to interact with a wide range of receptors and effectors. Western blot analysis using antibodies against the various G (GTP-binding) subunits indicate the existence of several 52–59 kDa G-proteins in both slender and stumpy forms of *T. brucei*. We have also found the induction of a 35/36 kDa G-protein in stumpy parasites. These proteins were confirmed to be G-proteins by their ability to specifically bind 8-azido GTP. The study of G-proteins in African trypanosomes may lead to the identification of associated receptor and effector molecules.

Endocytosis: We have developed cell-free approaches to investigate the individual steps of the endocytotic pathway in trypanosomes. Using horse radish peroxidase (HRP), we have demonstrated (in *T. brucei)* the uptake of this enzyme into flagellar pocket and endosome-like organelles which had been fractionated on both Percoll and sucrose gradients. We have also confirmed and extended these findings using Free Flow Electrophoresis (FFE). In addition to separating flagellar pocket from the more positively charged endosome compartment, FFE has proved useful in fractionating lysosomes as well as putative cis- and trans-Golgi membranes. Of the 4–5 galactosyl transferase positive peaks resolved, only one contained acid phosphatase, a marker known to localize in the trans-Golgi region. This enzyme was also found to be tartrate sensitive and was capable of utilizing inositol phosphate substrates suggesting that it could play a role in VSG-recycling. Antibodies have been produced against both Golgi and lysosome membrane fractions, as well as against flagellar pocket, endosomes and putative cis- and trans-Golgi membranes. These antibodies should prove useful reagents for the analysis of the endosomal system and provide immunologic clues in identifying useful trypanocidal antigens.

TR2.3 Characterization of enzymes which may be associated with the endocytotic pathway of trypanosomes

Scientists: J.D. Lonsdale-Eccles, D.J. Grab
Research Associate: G.W.N. Mpimbaza
Technician: S. Wasike
Student: T. Omalokoho
Supervisors: J.D. Lonsdale-Eccles, D.J. Grab

We have shown that, at permissive temperatures, proteins are endocytosed by African trypanosomes where they are presumed to be broken down by lysosomal proteases. A cysteine protease has been purified from *T. congolense* by affinity chromatography using cystatin-Sepharose. Antiserum was prepared against the purified enzyme and used to define the subcellular localization of the enzyme in *T. congolense*. It was shown to be present in both the flagellar pocket and endosomal/lysosomal system of the parasite. This system of the parasite, which comprises about 20% of the cell volume of *T. congolense*, is much larger in *T. congolense* than *T. brucei*.

Acid phosphatases are also found within the endosomal/lysosomal system of African trypanosomes. Using p-nitrophenylphosphate, beta-glycerolphosphate and beta-naphthylphosphate as substrates, we have identified two distinct acid phosphatases from *T. congolense* that may form part of this system. These enzymes have been partially purified and have been shown to have acid pH optima with the above substrates and may exist in multiple forms of different molecular weight.

TR2.4 Identification and characterization of invariant surface molecules of African trypanosomes

Scientists: D. Nandan, J.D. Lonsdale-Eccles, T.W. Pearson*
Research Associate: C. Wells
Technicians: D. Ndegwa

*Department of Biochemistry and Microbiology, University of Victoria, Victoria, B.C., Canada

A relatively simple technique for the purification of *Ricinus communis* Agglutinin I (RCA) binding proteins from *T. b. brucei* has been developed. Crude membranes from *T. b. brucei* were used as the starting material for the purification of RCA binding proteins. The crude membranes were solubilized in lysis buffer containing Triton X-100 and a cocktail of protease inhibitors. The solubilized material was incubated with biotinylated RCA. Biotinylated RCA binding proteins were recovered by immobilized streptavidin. The specific RCA binding proteins were released using galactose. SDS-PAGE anlysis of this preparation revealed proteins relatively free from VSG. These RCA binding proteins are currently being used to immunize BALB/c mice for the purpose of monoclonal antibody production.

The mannose-6-phosphate receptor exists in two forms in mammalian cells mainly concerned with lysosomal targetting. The dominant receptor is a glycoprotein

of molecular mass 215 kDa, the other one is smaller (46 kDa) and requires divalent cations for its efficient binding. A well characterized antibody against bovine cation independent mannose-6-phosphate receptor (a gift from Dr. S. Kornfeld, Washington University School of Medicine) showed reactivity with *T. b. brucei* proteins. Further, ß-glucuronidase was used as a ligand in affinity chromatography to identify the presence of mannose-6-phosphate receptor in trypanosomes. The cells were labelled with ^{35}S methionine, washed with PBS and used to prepare crude membranes. The solubilized membrane proteins were incubated with ß-glucuronidase-agarose. After extensive washing with low and high ionic strength buffers, mannose-6-phosphate binding proteins were released by mannose-6-phosphate. SDS-PAGE analysis of released proteins showed a major band in the region of 40 kDa.

TR2.5 Vesicular membrane glycoprotein of *T. vivax*

Student: B. Burleigh
Research Associate: C. Wells.
Supervisors: P. Gardiner, M.W. Clarke*

*Department of Microbiology and Immunology, University of Western Ontario, Canada

A monoclonal antibody (MAb) has been raised that recognizes a 65 kDa antigen on Western blots which is present in all *Trypanosoma vivax* isolates tested and in all life cycle stages of the parasite. The MAb does not recognize any proteins in *T. congolense* or *T. brucei*.

Biochemical studies suggest that the 65 kDa antigen is a glycoprotein with a hybrid type oligosaccharide chain. This has been demonstrated by its ability to bind to, and specifically elute from, Concanavalin A-Sepharose as well as *Ricinus communis* I agarose. It is also sensitive to endoglycosidase H digestion. After subcellular fractionation of bloodstream form parasites, the 65 kDa protein is found in membrane rich fractions. Separation of the 65 kDa protein into the detergent phase of Triton X-114 demonstrates its hydrophobic nature. Protease protection experiments employing a membrane fraction of *T. vivax* demonstrated the presence of the protein on the lumenal side of the membrane. Collectively, these data suggest that the 65 kDa protein is a membrane glycoprotein.

Immunolocalization studies using the monoclonal to label sections of Lowacryl-embedded *T. vivax* bloodstream forms show labelling associated with the membrane of tubular/vesicular organelles in the posterior portion of the parasite. Endocytosis of BSA gold and co-localization studies using the MAb show that the vesicles containing the 65 kDa protein occasionally contain endocytosed gold. The highest degree of co-localization is observed after allowing trypanosomes to ingest BSA-gold for 5 minutes.

The MAb identifies a 65 kDa glycoprotein (gp65) which is associated with the membrane of vesicles that are involved in endocytosis, and it may therefore be useful as a marker of an endocytotic compartment in *T. vivax*.

TR2.6 3′ Nucleotidase of *Trypanosoma vivax*

Student: B. Burleigh
Research Associate: C. Wells
Technician: R. Thatthi
Supervisors: P. Gardiner, M.W. Clarke*

*Department of Microbiology and Immunology, University of Western Ontario, Canada

The accumulated evidence of laboratory studies suggests that *T. vivax* is generally a fragile organism which may be attributable to a less dense coat structure and, perhaps, its greater ATP requirement. The potential exposure of underlying surface molecules in this species would seem to hold out greater hope for an immunological intervention, based on cell surface but invariant molecules than with *T. brucei* or *T. congolense*.

As a target invariant antigen we have selected a 3′ nucleotidase. This enzyme has been shown (by ourselves and others) to have an externally oriented cell surface disposition in *T. rhodesiense*, *L. donovani* and *C. luciliae*. The greater fragility of *T. vivax* has not yet allowed the certain demonstration of the enzyme electron microscopically in this species. However, it can be demonstrated as a Co^{2+} enhanceable activity in *T. vivax* by tube assay, and two gel assays dependent upon either the 3′ nucleotidase or nuclease activities of the enzyme. The enzyme activity from *T. vivax* runs under both reduced and unreduced conditions at a molecular weight of 35 kDa. *Leishmania donovani* samples run in the same gels exhibit molecular weights of 43 kDa, as previously published. We have raised a monoclonal antibody which reacts with a *T. vivax* molecule of the same molecular weight in immunoblots—but we have no direct evidence at this time that the antibody recognizes the enzyme. However, it has been shown that an anti-*Leishmania donovani* membrane serum reacts with a 35 kDa molecule in immunoblots of *T. vivax* antigen. Antibody select procedures to purify polyclonal anti-3′ nucleotidase antibodies from the anti-leishmanial serum are being pursued for the purification and identification of the 35 kDa antigen in *T. vivax*.

TR2.7 Biochemical and molecular biological characterization of the vacuolar ATPase of African trypanosomes

Scientists: W.R. Fish, N.B. Murphy, R.L. Cross*, E. Bramhall*
Research Associates: M.D. Macklin, C. Wells
Technicians: C.W. Muriuki, A.M. Muthiani
Student: W.A.M. Kitungu
Supervisor: W.R. Fish

*State University of New York, Syracuse, NY, USA

Biochemical studies—All previous studies undertaken with *T. b. brucei* have been repeated with *T. congolense*. Preparation of a presumptive endosomal fraction is

equally facile with *T. congolense* and the biochemical properties of the enzyme are very similar to those of *T. b. brucei* except IC_{50} values for N-ethylmaleimide and bafilomycin A_1 are slightly lower with the former parasite. Recent work has shown that the *T. congolense* enzyme contained in the membrane preparation: 1) is very sensitive to the arginine-specific reagent phenylglyoxal, 2) shows endoproteinase lys C/trypsin digestion patterns significantly different from the beef heart F_0F_1-ATPase, 3) can be labelled, albeit at low levels, with ^{32}P-azido-ADP, 4) shows bands of the appropriate size for the A, B, proton pore and accessory subunits on SDS-PAGE gels but with contamination from presumptive VSG and other proteins, probably of endosomal origin.

Molecular biological studies—The cDNA sequence of the open reading frame (ORF) of the A (catalytic) subunit from *T. congolense* has been almost completely determined. The polymerase chain reaction (PCR) has recently yielded specific, internally consistent products from lambda gt11 cDNA clones from a *T. congolense* metacyclic library that will allow completion of the 3′ region of the ORF. PCR products obtained from whole DNA from the same library using mini-exon and ORF primers show that the previously described 193 bp 5′-untranslated region is most likely immediately preceeded by the mini-exon. These results together suggest the transcript may well exceed 3.9 kbp. Sequence-specific primers have cleared all remaining sequencing anomalies. Among others, significant differences exist between the trypanosome subunit and that from other organisms: 1) in cysteine content, 2) at the Mg^{2+}-binding site, and, 3) at the noncatalytic, adenine nucleotide binding site. Although final assessment is incomplete, an ORF internal, 1.4 kbp Xba I fragment has been inserted into the pAX4b expression vector. Northern blots to estimate the size of the mRNA transcript for the A subunit have been unsuccessful with both random primed and nick translated probes using the 1.4 kbp Xba I fragment.

Previously described cDNA and other clones from the B (regulatory) subunit have been partially sequenced to determine their relationship. This process has yielded all but 30 amino acids of the complete ORF.

The PCR was unable to produce positive results for the membrane bound proteolipid proton pore subunit. A synthetic oligonucleotide based upon known sequences has allowed isolation of eleven cDNA clones which await analysis.

TR2.8 Possible chaperone-like molecules of African trypanosomes

Scientists: D. Nandan, E. Authié, T.W. Pearson*
Research Associates: C. Wells
Technicians: D. Ndegwa

*Department of Biochemistry and Microbiology, University of Victoria, BC, Canada

Affinity chromatography using gelatin (a denatured form of collagen)-Sepharose was used in a simple one-step procedure to purify two constitutively expressed proteins from African trypanosomes. Their ability to bind to a natural, denatured protein and to newly synthesized polypeptides of trypanosomes, together with their ATP-facilitated dissociation, their apparent molecular mass (68 kDa and 72 kDa) and their constitutive

expression in *Trypanosoma brucei brucei, T. congolense* and *T. vivax* indicated that these proteins are trypanosome molecular chaperones. One of the proteins (68 kDa) reacted with a monoclonal antibody which also reacts with members of a family of 70 kDa heat shock proteins (Hsp) from a wide variety of species. The other protein appears to be identical to a trypanosome protein which is an immunodominant antigen in cattle infected with *T. congolense.*

TR2.9 Trypanosomal Proteases

Scientists: J.D. Lonsdale-Eccles, W.R. Fish, E. Authié, E. Shaw*, J. Kornblatt†
Research Associates: G.W.N. Mpimbaza, I.D. Gumm, C. Wells
Technician: S. Wasike
Student: Z.R. Mbawa
Supervisors: J.D. Lonsdale-Eccles, W.R. Fish

* Freidrich Miescher-Institute, Basel, Switzerland
† Concordia University, Montreal, Canada

A major developmentally regulated cysteine protease from *T. congolense* has been isolated by affinity chromatography and its biochemical properties determined. It resembles the mammalian enzyme cathepsin L except that, unlike cathepsin L, it is stable and active at neutral and alkaline pH values and is activated by a molecule from serum. Several peptidyl diazomethanes, which are irreversible inhibitors of cysteine proteases, are potent inhibitors of this trypanosomal cysteine protease. They are also trypanolytic to bloodstream forms of *T. congolense* and *T. brucei* and to epimastigote forms of *T. vivax*. Approximately 50–60% of the cDNA corresponding to a cysteine protease of metacyclic *T. congolense* has been sequenced and it shows extensive homology to the sequence of a similar cysteine protease from *T. brucei*. The protease is found in the endosomal/lysosomal system of the parasite and has an acid pH optimum. It is also found in the flagellar pocket and on the surface of the parasites.

A soluble, cytosolic enzyme that is released from isolated trypanosomes has been purified from *T. brucei* and from *T. congolense*. The enzyme has defied classification because of its unusual biochemical characteristics. Detailed kinetic analysis has been performed on the enzyme from *T. brucei*. The results show that the specificity of the *T. brucei* enzyme is characteristic of pro-hormone processing enzymes of mammalian cells. Immunoelectron microscopic analysis using a polyclonal antiserum to the *T. brucei* enzyme support the cytosolic localization of the enzyme. Monoclonal antibodies are being prepared.

TR2.10 *Trypanosoma vivax:* sequence analysis of a bloodstream form variable surface glycoprotein

Scientists: P.R. Gardiner, V. Nene, S. Kieland*
Technician: R. Thatthi
Student: B. Burleigh
Supervisors: P. Gardiner, M.W. Clarke†

*Protein Microsequencing Facility, University of Victoria, BC, Canada
†Department of Microbiology and Immunology, University of Western Ontario, Canada

A cDNA library prepared from ILDat 2.1, a clone of a stock of *T. vivax* from Lugala, Uganda, was screened with a polyclonal rabbit serum raised against the ILDat 2.1 VSG. The complete nucleotide sequence of one of the positive clones was determined. N-terminal and an internal fragment sequence were confirmed by amino acid analysis and sequencing of unmodified VSG and cyanogen bromide digested VSG peptides. The deduced molecular weight of the total VSG is 40 kDa making it one of the smallest VSGs described. The *T. vivax* VSG showed no primary sequence homology to published sequences of VSGs from other pathogenic trypanosomes. The VSG incorporates ^{3}H-myristate and ^{3}H-mannose suggesting, like VSGs of other trypanosomes, that the *T. vivax* is a glycoprotein bearing a fatty acid tail but as the Mr of the VSG in SDS-PAGE is the same as that of the deduced protein sequence, the extent of glycosylation is not thought to be great.

Previous studies have indicated the existence of isoVATs in *T. vivax*, contributing to the serological cross reactivity amongst isolates from West Africa. The ILDat 2.1 parasite shows similarities to the West African group but the labelled ILDat 2.1 VSG gene, or an oligonucleotide within the 5 prime fragment of the gene, only hybridized with DNA from parasites from Lugala and Teso in Uganda, but not with parasites from Kenya, Nigeria, Côte d'Ivoire or The Gambia.

TR2.11 Comparative analysis of *T. congolense* VSG expression sites

Scientist: P. Majiwa
Technicians: M. Mbugua, A. Muthiani
Student: N. Doshi
Supervisors: P. Majiwa, N.K. Olembo*

*Biochemistry Department, University of Nairobi, Kenya

We have previously analysed several bloodstream form-specific VSG genes of *T. congolense* clones derived from ILNaR 2 and ILNaR 3. More recently, we have completed the primary analyses of two different metacyclic form stage-specific transcripts encoding mVSG 1 and mVSG 2 of IL 3000, a clone of ILNaR 2.

The predicted amino acid sequences of both mVSG 1 and mVSG 2 were significantly similar only to VSGs of *T. congolense* YNat 1.1 and YNat 1.3. Direct

comparison of the deduced amino acid sequences of the antigens encoded by each of the cDNA clones with each other, and with those of two bloodstream form VSGs of *T. congolense*, revealed significant conservation of structural domains. Notable were a heptapeptide which was present only in the two metacyclic VSGs, and a tripeptide conserved in the central domain of all four antigens. The conservation of these amino acids is particularly interesting given the absence of any immunological data indicating the existence of isotypic VATs among different isolates, or serodemes, of *T. congolense*.

Also observed was the occurrence of a doublet of amino acids, leucine and cysteine, the same distance from an asparagine conserved in all the four antigens. These amino acids have been demonstrated to indicate the proximity of the amino-terminal end of mature VSGs of both *T. congolense* and *T. brucei*. Indeed, the N-terminal ends of mature YNat 1.1 and YNat 1.3 were shown to be four and five amino acid residues, respectively, from the conserved asparagine.

TR2.12 Gene regulation, rearrangement and amplification during cyclical development of *Trypanosoma congolense*

Scientist: P.A.O. Majiwa
Technician: M. Mbugua

Various types of repetitive DNA sequences in the African trypanosomes are involved in the movement of specific genes between different chromosomes. We have identified two different cDNA clones which contain the transcripts of a *Trypanosoma congolense* gene that is regulated during development. The gene is transcribed specifically in the bloodstream form stage of the parasite, irrespective of the VSG expressed. Each of the cDNAs is composed of transcripts that originate from at least two different genomic loci, one of which has a DNAse I sensitive chromatin structure. A locus homologous with that which donates a part of each of the cDNAs is differentially amplified and rearranged in different cloned isolates of *T. congolense* from diverse antigenic repertoires. Neither locus is apparently linked to any of at least three different VSG genes expressed in two distinct antigenic repertoires. Aspects of this gene that are being investigated include, 1) how the transcripts from the two loci are spliced post-synthesis, 2) the regulation of its transcription during development of the parasite through the different life cycle stages, and, 3) the mechanism of its rearrangement and differential amplification. These may provide information regarding the developmental biology of *T. congolense*.

TR2.13 The identification of virulence genes in *T. b. brucei*

Scientists: N.B. Murphy, H. Hirumi
Research Associate: K. Hirumi
Technicians: P. Pandit, S. Wasike

A number of attenuated GUTat 3.1 *T. brucei* mutants have been selected by a series of manipulations in *in vitro* culture systems. These parasites have a normal doubling time in culture, but fail to establish a detectable parasitaemia when used to infect mammalian hosts with up to 1×10^7 parasites. Karyotype analysis previously identified a deletion in an intermediate sized chromosome in an attenuated derivative, but this deletion was not consistently observed in independently derived mutants. More recent analyses have identified an apparently consistent alteration involving two chromosomes of approximately 1.5 megabases in size. Transfection of total DNA from a virulent ILTat 1.1 parasite resulted in three independent revertants whose chromosome profile appeared identical to the parental attenuated population. Several DNA probes have been used in an attempt to identify the region of the genome which has undergone an alteration in these parasites without success. Other studies have included a comparative analysis between labelled proteins from an attenuated parasite and the virulent parental isolate by 2-D polyacrylamide gel electrophoresis. Some qualitative and quantitative differences in the protein profiles between these populations were observed.

TR2.14 Miniexon-derived RNA and the control of gene expression control

Scientists: R. Pelle, T. Pearson*, N. Murphy
Research Associate: N. MacHugh
Technician: S. Wasike

*Department of Biochemistry and Microbiology, University of Victoria, BC, Canada

In trypanosomes, the mini-exon or spliced-leader sequence of 39 nucleotides (nt) is found on all mRNAs and is thought to be important in the translation process in these parasites. The mini-exon is spliced onto RNA transcripts by a trans-splicing mechanism through a mini-exon derived (med) RNA transcript of approximately 145 nt. We have found that, 1) Some medRNA transcripts are polyadenylated in non-dividing bloodstream forms of *T. b. brucei*, 2) That medRNA transcripts are abundant in the cytoplasm as well as the nucleus, and, 3) The medRNA transcript is associated with cytoplasmic proteins, some of which may vary in different life cycle stages of the parasite.

The central role that medRNA plays in the expression of all trypanosome genes makes it a potential target for attack in the control of trypanosomiasis. Regulatory proteins associated with medRNA have been identified through the use of UV cross-linking. This study has been extended to the identification of regulatory cytoplasmic proteins which bind to VSG mRNA, which may be effectors involved in the control of VSG expression. The study of effectors of VSG expression was stimulated by the

observation that differential processing of VSG may occur in dividing and non-dividing bloodstream forms of *T. b. brucei*.

TR2.15 The identification of genes and gene products involved in trypanosome cell division

Scientists: N.B. Murphy, B. Ricard, P. Leegwater, H. Hirumi, J.C. Mottram*, A. Tait*, K. Crawford†, P. Nurse†, E. Pays§
Research Associates: E. Gobright, C. Wells
Technicians: P. Pandit, S. Wasike, A. Muthiani

*Wellcome Unit of Molecular Parasitology, University of Glasgow, Glasgow, UK
†ICRF Cell Cycle Group, University of Oxford, Oxford, England
§Departement de Biologie Moleculaire, Universite Libre de Bruxelles, Brussels, Belgium

Four classes of cell division cycle (cdc) genes are currently under study in several trypanosomatid species, 1) Members of the *cdc*2 gene family are being studied in four species of trypanosomes, *L. mexicana* and *T. brucei* at Glasgow and *T. congolense* (both Kilifi and Savannah) and *T. vivax* at ILRAD, 2) The cyclins of *T. brucei*, whose association with the *cdc*2 gene product in other systems determine the timing of entry into the cell division cycle, are the subject of studies being carried out by Dr. Pays' group in Brussels, 3) DNA polymerase alpha (*polα*) from *T. brucei* and *T. vivax* and an associated gene from *T. brucei* are being studied at ILRAD in collaboration with Utrecht, and, 4) Cytoskeletal genes from *T. brucei* and *T. congolense* are also being studied at ILRAD.

For all classes of cdc genes being studied, potential homologues have been identified and their DNA sequences have been partially or completely deduced for the different species mentioned above. For *T. congolense*, two potential *cdc*2 homologues have been identified, one of which has been focused on because of its greater similarity to *cdc*2 from other species. This homologue has also been identified in *L. mexicana* and *T. brucei* by the Glasgow group. An unusual feature of the deduced amino acid sequences from these species is the presence of the sequence PCTAIREIS in place of PSTAIREIS, which is characteristic of *cdc*2 kinases. The unusual substitution of cysteine for serine in this sequence led to the further examination of this sequence in two additional trypanosome species, *T. vivax* and the Kilifi sub-species of *T. congolense*. For all species of trypanosome examined, this unusual substitution is conserved. Attempts to complement a *S. pombe* temperature sensitive *cdc*2 mutant with both the *L. mexicana* and *T. congolense* genes have been unsuccessful.

Antisera raised against fusion proteins from these genes and to peptides from the deduced amino acid sequences consistently detect 2 proteins of ~34 kDa and ~65 kDa in all life cycle stages of *T. congolense*, suggesting that a closely related family of *cdc*2-like proteins exist in *T. congolense* whose members are expressed throughout the life cycle. Similar results have also been obtained for *L. mexicana*. Homologous

transcripts are also detected in all life cycle stages of *T. congolense* without significant variation.

Studies on the *polα* gene of *T. brucei* have now been extended to *T. vivax* by the identification of clones containing part of the gene sequence. Attempts to isolate the *T. congolense* homologue have not been successful. In *T. brucei*, an associated gene of unknown function appears to be co-transcribed with the *polα* gene, but is constitutively expressed in dividing and non-dividing bloodstream forms while *polα* transcription is regulated. The close linkage of the associated gene is conserved in *T. congolense* as determined by Southern blot analysis, but has not been verified for *T. vivax*. Antisera have been raised to fusion proteins from the *T. brucei polα* gene and this antiseum has been used to show nuclear localization of the encoded product in dividing, but not in non-dividing trypanosomes. This antiserum should be a useful marker for trypanosomes which are going through the S-phase in the cell division cycle.

Several cytoskeleton associated proteins have been identified and some of their genes characterised as described in another report (see abstract TR2.18). A gene family coding for proteins of ~17 kDa which co-purified with the cytoskeletal proteins has also been characterised. These genes are of particular interest due to their high level of conservation between different trypanosome species (>90% homology in the 5′-DNA coding sequences between *T. brucei* and *T. congolense)*. This high level of conservation between trypanosome species implies severe constraints on the divergence of these sequences. However, a significant amount of divergence between *T. brucei* and *T. congolense* is evident at the 3′ end of these genes. It is clear that different selective mechanisms are acting at the different domains of these genes in these trypanosome species. The *T. brucei* gene family can be divided into two linked groups which also differ at their 3′ end. One group is differentially expressed in dividing and non-dividing bloodstream forms of *T. brucei*. Although the function of these genes has not yet been determined, a search of the protein database has revealed significant homology to the partial amino acid sequence of a phosphorylated 19 kDa mammalian protein whose expression is increased in certain transformed cell lines.

TR2.16 Trypanosomal kinases and phosphatases

Scientists: O.K. ole-MoiYoi, J.D. Lonsdale-Eccles, D.J. Grab, W.R. Fish, T. Tsukamoto*
Research Associates: G.W.N. Mpimbaza, M.D. Macklin, E. Gobright
Technicians: Y. Verjee, S. Wasike

*Department of Applied Biological Sciences, Saga University, Japan

By studying phosphorylation changes in the proteins of slender and stumpy bloodstream forms of *T. brucei*, we have shown that such changes may be important in the regulation of these parasites. The predominant phosphorylation events occur on serine and threonine residues. We have isolated and partly characterized three distinct protein kinases from *T. brucei* that have some biochemical properties of casein kinase enzymes of higher eukaryotes.

We have also purified casein kinase II from bovine brain. The enzyme has the expected subunit structure. This enzyme will be compared with trypanosomal kinases some of which are being purified. One of the trypanosomal CK-II-like enzymes we have identified has a heterotetrameric composition of alpha/alpha'/beta2. Its functional properties are similar but distinct from their mammalian counterparts. In addition, we have identified the presence of protein phosphatase activity in *T. brucei* that may be involved in counteracting the activity of the kinases.

TR2.17 Developmental regulation of phosphotyrosine-containing and autophosphorylating proteins in *Trypanosoma (Nannomonas) congolense*

Scientists: M. Parsons*, W.R. Fish
Technicians: A.M. Muthiani

*Seattle Biomedical Research Institute, Seattle, WA, USA

Tyrosine phosphorylation of proteins and autophosphorylation of presumptive protein kinases, implicated in cell cycle control and developmental regulation in other eukaryotes, are potentially important areas in which African trypanosomes may show stage specific regulation. *T. congolense* IL 3000 bloodstream, procyclic, epimastigote and metacyclic forms were prepared for SDS-PAGE and subsequent Western blotting for the assessment of the presence of these moieties or processes.

Western blots of the above material, probed with an anti-phosphotyrosine antibody, showed dramatic life cycle stage-specific differences. Of particular interest are, 1) an ~110 kDa band predominant in metacyclics, 2) ~54 and 72 kDa bands present in diminishing amounts in bloodstream, procyclics and epimastigotes, respectively, but undetectable in metacyclics, and, 3) ~50 and 68 kDa bands found only in insect forms (procyclics, epimastigotes and metacyclics).

Kinase renaturation assays performed with the same trypanosome preparations also showed life cycle stage-specificity, many of the differences being subtle. Interestingly, bloodstream and metacyclic forms (both mammal infective forms) were qualitatively similar, except for the complete absence, in bloodstream forms, of a ~50 kDa band found in all other life cycle stages. Epimastigotes were most distinctive, showing a strong band at ~28 kDa, while other life cycle stages showed little (procyclics and metacyclics) or no (bloodstream forms) activity. The latter observation must be confirmed, since experiments on other epimastigote populations showed variation.

These experiments show that, 1) phosphorylation of tyrosine residues occurs in proteins in all life cycle stages of *T. congolense*, 2) tyrosine phosphorylation shows stage specificity, 3) protein kinases (shown by autophosphorylation) of a variety of Mr are present in all life cycle stages, and, 4) patterns of autophosphorylation are correlated with stage specificity.

TR2.18 Identification and characterization of cytoskeletal proteins of *T. congolense*

Scientists: O.K. ole-MoiYoi, N. Murphy, J.D. Lonsdale-Eccles, R. Hamers*
Research Associate: C. Wells
Technician: S. Wasike
Student: W.O. Endege
Supervisors: O. ole-MoiYoi, R. Hamers*

*Institut voor Moleculaire Biologie, Vrije Universiteit Brussel, Belgium

The isolation and characterization of a *T. congolense* cytoskeletal protein doublet, Tp80, has previously been reported. The antibodies to these proteins also reacted with other proteins of Mr 53 kDa (Tp53) and 17 kDa (Tp17). Antibody-select using clones expressing Tp80 was achieved. The selected antibody was employed in immunoelectron microscopy and it identified an antigen in the paraflagellar rod. The Tp80 doublet of proteins are phosphorylated by casein kinase-like enzymes of *T. brucei*. One of these proteins appears to diminish as *T. brucei* undergoes the transition from slender to stumpy bloodstream forms.

Two cDNA clones encoding the *T. congolense* doublet of proteins of approximate Mr 80 kDa were identified and have been completely sequenced. The deduced amino acid sequences revealed 63% homology and are similar to recently described paraflagellar rod protein sequences of *T. brucei*. The two *T. congolense* and *T. brucei* genes are differentially expressed. The predicted tertiary structure of the proteins suggests a high degree of helicity throughout both molecules, both of which are of paraflagellar rod localization.

The third cytoskeletal protein of *T. congolense*, with a tentative localization to the region between the kinetoplast, basal bodies and the flagellar pocket of trypanosomes, has been identified and termed Tp53. The cDNA for this protein has been isolated and is currently being further characterized.

These proteins are related and are encoded by a family of genes which are detectable in all trypanosomal DNAs so far examined.

TR3.1 Identification and mapping of bovine polymorphic genetic markers

Scientists: S.J.Kemp, L.Brezinsky, A.J. Teale
Technicians: J.Mwakaya, J.Wambugu

Highly polymorphic dinucleotide repeats (termed microsatellites) in the bovine genome have been located in two ways. A computer search of published sequences yielded a number of regions containing dinucleotide repeats. PCR primers were designed to amplify these regions and four useful polymorphic markers were identified. These have been applied to large full sibling families and Mendelian inheritance demonstrated. A genomic library was also made and screened with a poly CA oligonucleotide. Sixteen potential microsatellite sequences were identified. PCR primers have been made for 7 of these and 4 give polymorphic products. We can thus expect approximately 8 useful markers from this library. A new library is presently under construction.

A computer programme has been written to aid in the crucial step of PCR primer design. It is proving valuable in identifying primers which give a clean PCR product under a range of amplification conditions. This will be important when multiplex PCR is used.

The use of short (10 nucleotide) random oligonucleotides for fingerprinting by means of randomly primed PCR (RAPD-PCR) has been attempted. It appears to reveal polymorphisms in cattle but the 'robustness' and usefulness of this approach remains to be demonstrated.

TR3.2 Studies of population genetics of N'Dama and other African native cattle

Scientists: R. Suzuki, S. J. Kemp, A.J. Teale
Technician: J. Mwakaya

Eleven biochemical polymorphisms have been detected in N'Dama and Boran breeds and they have proved to be useful genetic markers for bovine genome mapping.

Biochemical polymorphisms were also employed for a phylogenetic study of African native cattle breeds. Three hundred and twenty-eight blood samples of 13 populations (4 Gambia N'Dama populations, 3 Gabon N'Dama populations, 6 East African Zebu populations) were analysed and from their allele frequency data Nei's genetic distances were calculated.

Mitochondrial DNA studies were performed to obtain information on maternal phylogenetic relationships among African native cattle breeds. Whole mitochondrial DNA purified from the blood samples of 3 N'Dama and 3 Boran cattle were analysed using the cleavage patterns for 7 enzymes by which polymophisms were detected in recent studies. The cleavage patterns showed no polymorphism.

PCR products of D-loop and URF5 regions from 58 samples (Gambia N'Dama 13, Gabon N'Dama 12, Friesian 8, Boran 13, Maasai Zebu 12) were digested by restriction enzymes and cleavage patterns were analysed. UFR5 samples were digested by 7 enzymes and only one animal showed a divergent pattern in one digestion (Eco R 1). D-loop samples were digested by 12 enzymes and 5 showed polymorphism, but

only one animal showed a divergent pattern in each enzyme digestion. Thus, 5 animals showed 5 different restriction enzyme maps and the other 53 animals all showed identical patterns.

TR3.3 Mapping of trypanotolerance genes in an N'Dama × Boran resource population

Scientists: A. Teale, D. Kennedy, S. Kemp
Research Associates: S. Leak, S. Minja
Technician: J. Ngatti

Two full sibling families, each of more than 30 individuals, of N'Dama × Boran F1 animals have now been constructed using the techniques of multiple ovulation and embryo transfer. The two families have been derived from two N'Dama bulls and two Boran cows. Work continues on the production of two more families of similar size using two additional bulls and cows.

To date 39 F1 animals of four families have been challenged with *Trypanosoma congolense* clone IL 1180 and 28 have been monitored for the planned observation period post challenge. Challenges have been performed at one year of age with the bites of five infected tsetse flies *(Glossina morsitans centralis)*. Animals were challenged on a monthly basis as they became available. The first three challenges in January, February and March 1991 included a total of ten Boran controls.

Animals were monitored regularly both before challenge and for a minimum of 80 days post challenge. Parameters scored were parasitaemia, packed red cell volume percent, total and differential leukocyte counts, antigenaemia, serological responses to invariant trypanosome antigens and bodyweight. Animals in which the PCV fell to 15% or below were treated to ensure recovery and removed from the challenge phase of the experiment.

PCVs were reduced in all F1 animals with very little variation in kinetics or degree. The effect on PCV was less marked in the F1s than in the Boran controls as evidenced by the fact that 2 of 28 F1 animals required to be treated before the end of the 80-day observation period compared with 6 of 10 Boran controls.

Falls in neutrophil and leukocyte counts were less marked in the F1s than in the Boran controls. Initial observations suggest little correlation between serological response to invariant antigens and degree of 'trypanotolerance' in the animals examined thus far.

The results of these challenges suggest that the F1 animals are relatively homogeneous in their response to challenge. They are more resistant to the effects of the defined challenge than the majority of Boran cattle but less resistant than N'Dama animals which have been examined previously (which include the founder bulls). The F2 generation will be produced by intercrossing the F1 animals between families. The results are encouraging as far as prospects for mapping of trypanotolerance genes in the F2 generation are concerned.

TR3.4 Cloning and characterization of bovine tumor necrosis factor

Scientists: O.K. ole-MoiYoi, M. Sileghem, L. Logan-Henfrey, B. Mertens, D. Williams
Research Associates: L. Gaidulis, M. Macklin, E. Gobright

In order to produce bovine tumor necrosis factor (TNF) for use in experiments with materials from cattle infected with trypanosomes, oligonucleotides based on nucleotide sequences of conserved amino acid domains of mammalian TNFs were constructed and used to probe Southern blots of bovine DNA that had been digested with a variety of restriction enzymes. Bovine polyA+ RNA was prepared from activated macrophages and used to prepare cDNA. PCR reactions were performed with this cDNA as template under a variety of conditions in an attempt to obtain fragments large enough for sequencing and for further cloning. Employing both of these approaches DNA fragments which hybridized to the original primers were cloned into vectors and are undergoing sequence analysis and further characterization.

TR3.5 Production of co-stimulatory cytokines by macrophages during infection with *T. congolense*

Scientists: M. Sileghem, J. Naessens
Technicians: R. Saya, K. Tikolo

The release of co-stimulatory cytokines by bovine monocytes and macrophages was analysed using a modification of the murine thymocyte co-stimulation assay. Bovine monocytes which were purified by adhesion to plasma-coated gelatin and cultured *in vitro* were found to release high titres of co-stimulatory cytokines following *in vitro* stimulation with bacterial lipopolysaccharide. In the absence of lipopolysaccharide, a spontaneous secretion was noted which was very low and usually remained below the detection limit of the assay. In contrast, monocytes derived from *T. congolense*-infected animals manifested a marked spontaneous secretion of co-stimulatory cytokines *in vitro*. In general, the titres were three- to four-fold higher than the titres in the supernatants from lipopolysaccharide-activated monocytes. This indicated that the cells were activated to release co-stimulatory cytokines by the infection with *T. congolense*. The high spontaneous secretion of co-stimulatory cytokines was noted with both blood monocytes and splenic macrophages. The activation was maintained throughout the infection, but at the end of the experiment, around week 6 to week 7 post infection, the titres returned to pre-infection values. However, at that stage, the cells were perfectly able to respond to an *in vitro* trigger with lipopolysaccharide. This indicated that the monocytes were not exhausted or unresponsive at the end of the experiment but were simply no longer activated by the infection.

A comparative analysis of 4 Boran and 4 N'Dama cattle infected with *T. congolense* revealed that the monocytes from the N'Dama cattle manifested a 16-fold higher secretion of co-stimulatory cytokines than monocytes from Boran cattle one week after infection. At later stages of infection the titres were comparable in both

breeds. At the end of the experiment, the titres had returned to pre-infection values in both Boran and N'Dama cattle.

TR3.6 Role of macrophage activation in the pathology of *Trypanosoma congolense* infection

Scientists: M. Sileghem, L. Logan-Henfrey, D.J.L. Williams
Technician: K. Tikolo

Tumor necrosis factor α(TNFα), a cytokine which is mainly produced by cells from the monocyte/macrophage lineage, is known to play a crucial role in the induction of anaemia associated with experimental murine malaria. At present, it is not clear whether TNFα has a similar role in induction of anaemia during African trypanosomiasis in cattle. To study the involvement of TNFα in the pathology of the disease, monoclonal antibodies specific for bovine TNFα have been developed in mice. The monoclonal antibody BC9 was found to block the capacity of bovine TNFα to lyse L929 tumour target cells *in vitro*. Large quantities of this neutralizing antibody have been produced which will be used for treatment of cattle. The monoclonal antibody BC9 has also been found to be suitable to detect TNFα using a radio-immune assay and a sandwich ELISA assay. Other monoclonal antibodies such as BC7 have been used to detect intracellular TNFα in lipopolysaccharide-activated monocytes using flow cytometry with saponin-permeabilized cells. This technology will be applied to analyse TNFα production in N'Dama and Boran cattle throughout infection with *T. congolense*. Finally, TNF-specific monoclonal antibodies which do not interfere with the binding of TNFα to its receptor will be used to measure expression of receptors for TNFα.

TR3.7 Immunosuppression during bovine trypanosomiasis

Scientists: M. Sileghem, J.N. Flynn
Technician: R. Saya

Lymph node cells (LNC) from Boran cattle infected with *Trypanosoma congolense* were found to be unable to proliferate *in vitro* in response to a mitogenic trigger. This unresponsiveness was attributed to the presence of suppressive cells which interfered at the level of both interleukin 2 (IL-2) secretion and interleukin 2 receptor (IL-2R) expression. Depletion of the macrophage fraction through fluorescence-activated cell sorting abrogated suppression of both IL-2 secretion and IL-2R expression whereas inhibition of prostaglandin synthesis and degradation of hydrogen peroxide had no effect.

To analyse the involvement of soluble factors in immunosuppression, co-cultures were set up where LNC from an infected animal were separated from LNC from a normal animal by a porous membrane. The mitogen-induced proliferation of the normal LNC was severely suppressed in such co-cultures but could be markedly restored by addition of indomethacin which prevents the synthesis of prostaglandins.

However, in duplicate co-cultures where the cells were mixed, indomethacin had no effect. Hence, the suppression was due to the co-existence of at least two mechanisms, a prostaglandin-independent mechanisms which required cell-cell contact and a prostaglandin-dependent mechanism which did not require such contact.

Data from experiments with inbred mouse strains have shown that interferon c (IFNc) has a crucial role in the induction of prostaglandin-independent immunosuppression during African trypanosomiasis. The involvement of IFNc in the suppression observed during bovine trypanosomiasis was analysed through the addition of recombinant bovine IFNc. It was found that IFNc did not affect the mitogen-induced proliferation of normal LNC. However, when added to co-cultures where suppression was suboptimal, the observed immunosuppression was greatly enhanced. Since IFNc is known to trigger the release of hydrogen peroxide it is important that the enhancement of suppression was not affected by the addition of catalase.

TR3.8 Immunosuppression in N'Dama and N'Dama × Boran cattle following *T. congolense* infection

Scientist: J.N. Flynn
Technician: R. Saya

Tsetse-transmitted *Trypanosoma congolense* infection causes an impairment of T-cell function in Boran cattle. To assess the importance of this phenomenon in the ability of trypanotolerant cattle to control infection with trypanosomes, T-cell responses to mitogenic stimulus with Concanavalin A were assessed in N'Dama and N'Dama × Boran F1 cattle throughout infection.

Studies of the responses of peripheral blood mononuclear cells (PBMC) from Boran and N'Dama cattle revealed a similar pattern. Depressed T-cell responses were observed in both breeds 14 and 21 days after infection after which these responses returned to pre-infection levels. This effect was not observed in the N'Dama × Boran F1 cattle. The T-cell responses of these cattle remained the same throughout infection. A comparison was also made of PBMC prepared by defibrination using glass beads and using Alsever's solution as an anti-coagulant. No significant differences were observed. The FACS profiles of PBMC prepared in these two ways was also the same.

Immunosuppression was also studied in an MHC-matched pair of N'Dama cattle; one animal was infected whilst its MHC-matched partner served as a normal control. Lymph node biopsies were performed on both animals on day 21 following infection and the responses of the cells (LNC) to Con A examined. LNC from the infected animal failed to respond to the mitogenic stimulus. Co-culture experiments in which the cells from these nodes were mixed with LNC or PBMC from the normal MHC-compatible animal revealed the presence of suppressor cells, acting in a prostaglandin-independent manner, capable of arresting Con A-induced T-cell proliferation. These results suggest that *T. congolense* induced immunosuppression is unlikely to be one of the mechanisms involved in trypanotolerance.

TR3.9 Phagocytic potential of monocytes and macrophages during infection with *Trypanosoma congolense* in cattle

Scientist: M. Sileghem
Technician: R. Saya

The uptake of trypanosomes by phagocytes was studied through a two-colour immunofluorescence analysis where trypanosomes were labelled with fluorescein (green fluorescence) and phagocytes were labelled with phycoerythrin (red fluorescence). The emission of green fluorescence by red fluorescent cells was measured by flow cytometry and used as an indicator of parasite uptake. This method allows the quantification of parasite uptake by phagocytes without the requirement to purify the phagocytes or to sort the unbound parasites from the cell-bound parasites.

The uptake of trypanosomes was analysed with both granulocytes and monocytes. In both cases, an uptake of roughly 200 to 300 trypanosomes per 100 phagocytes was noted. No differences were observed between phagocytes from Boran cattle and N'Dama cattle. Following infection with *T. congolense*, only the granulocyte-enriched fraction was studied. The number of cells which were able to bind parasites was steadily increased from 50% pre-infection to 90% at week 6 post-infection. The number of trypanosomes bound per cell was not markedly affected and was marginally increased from 4 to 6 trypanosomes per granulocyte. Hence the total uptake of parasites was increased from 200 per 100 granulocytes to 550 parasites per 100 granulocytes. No differences were observed between Boran and N'Dama cattle.

As a control, the uptake of fluorescein-labelled bacteria was followed throughout infection. In contrast to the uptake of trypanosomes, the uptake of bacteria was not altered by the infection, but the oxidative burst induced by the uptake of the bacteria was increased during infection. Again, no differences were observed between Boran and N'Dama cattle.

TR3.10 The effect of peripheral blood leukocyte culture supernatants and recombinant cytokines on *in vitro* macrophage erythrophagocytosis

Student: V.O. Taiwo
Supervisors: D.J.L. Williams, L.L. Logan-Henfrey

An *in vitro* assay to measure erythrophagocytosis was established, using peripheral blood monocytes (PBM) and ^{51}Chromium-labelled erythrocytes. This assay was used to show that monocyte-mediated erythrophagocytosis increased in cattle during experimental *T. congolense* infection. The factors which trigger PBM to erythrophagocytose are not known. Therefore, in an attempt to identify molecules which could be involved in inducing erythrophagocytosis *in vivo*, trypanosome antigens and mammalian immunomodulatory factors were added to bovine PBM isolated from two uninfected cattle and levels of erythrophagocytosis measured.

Significantly higher levels of erythrophagocytosis were obtained using PBM incubated with whole *T. congolense* lysate for 18 or 48 hours compared to unstimulated

control PBM. In contrast bacterial lipopolysaccharide did not induce erythrophagocytosis.

Culture supernatants collected from peripheral blood mononuclear cells either 24 or 48 hours after stimulation with phytohaemagglutinin were also added to PBM. These lymphokine-rich supernatants induced levels of erythrophagocytosis above those in the control monocytes. Exposure of these supernatants to acid conditions (pH2) for 24 hours partially ablated their stimulatory activity. Human recombinant tumour necrosis factor and bovine recombinant interferon-gamma were also found to stimulate erythrophagocytosis.

These results show that both cytokines and parasite antigens can activate PBM to erythrophagocytose *in vitro*. It is possible that these molecules play a role in inducing erythrophagocytosis in trypanosome-infected cattle.

TR3.11 Early immune responses following tsetse-transmitted trypanosomiasis

Scientists: J.N. Flynn, D.J. McKeever, D. Mwangi
Technicians: R. Saya, K. Tikolo

In previous studies, following primary tsetse-transmitted infection with *T. congolense*, there was a profound impairment of T-cell function at the level of the lymph nodes in infected cattle. This impairment was associated with the presence of suppressor cells which acted in a prostaglandin-independent manner to down-regulate IL-2 production and IL-2 receptor expression. Depletion of the monocyte-macrophage population from the lymph node cells of infected cattle by fluorescence activated cell sorting resulted in a restoration of T-cell function, suggesting an important role for these cells in the development of immunosuppresion.

However, it was not clear whether these cells were being generated in the lymph node itself or were generated within the skin and travelling to the lymph node via the afferent lymphatic vessels to exert their effect. To answer this question and to examine the early immune events occurring at the tsetse bite site, the afferent lymphatic vessels (pseudo-afferent) of two Boran cattle were cannulated. Following cannulation the animals were infected with *T. congolense* by tsetse bites given within the drainage area of the cannulated lymphatic vessels. The changes in cell output were assessed by FACS analysis. Little change occurred in the output of CD2+ T-cells in the lymph, and within this population the CD4+:CD8+ ratio remained relatively constant. By contrast, a marked decrease was observed in the output of γδ T-cells over the first 7–8 days following infection. The number of sIgM+ B-cells appeared to increase and peaked at 3–5 days post-infection. Thereafter the level returned to pre-infection values. Little change was observed within the afferent lymph veiled cell (ALVC) population, although the number of these cells expressing IgM did increase following infection. This was presumably due to the adsorption of IgM free in the interstitial fluid onto the surface membrane of these cells.

The degree of activation and responsiveness of the lymphocytes present in the afferent lymph was assessed in two ways: First, the cells were examined for the expression of activation markers and, second, the cells collected each day were stimulated *in vitro* with the mitogen concanavalin A (Con A).

The number of lymphocytes expressing the IL-2 receptor α-chain decreased from day 5 post-infection. However, there was still a significant number of cells expressing this molecule. The amount (mean fluorescence intensity) of the IL-2 receptor expressed on individual cells did not alter throughout infection. The expression of Cact 116A or CD69, another activation marker, increased from day 5 post-infection, indicating that lymphocyte activation was occurring at this time. Afferent lymph cells collected pre-infection and on the first four days post-infection proliferated in response to stimulation with Con A *in vitro*. However, from day 5 this response to Con A was completely abrogated. Previous studies have shown that following infection, lymph node cells not only fail to respond to Con A themselves but also suppress the potential of normal lymph node cells or PBMC to respond to Con A. This was not the case with afferent lymph cells, and PBMC could proliferate normally in response to mitogens whilst in the presence of suppressed afferent lymph veiled cells.

These studies suggest that although a degree of lymphocyte activation occurs following tsetse-transmitted infection with *T. congolense*, this may be sub-optimal due to the immunosuppression which appears to operate at the level of the skin in addition to the lymph nodes.

TR3.12 Antigen-specific responses of T-cells during bovine trypanosomiasis

Scientist: J.N. Flynn
Research Associate: J. Magondu
Technician: R. Saya

Following primary tsetse-transmitted challenge with *T. congolense*, antigen specific T-cell proliferative responses were observed *in vitro* using mononuclear cells isolated from the peripheral blood and spleen of infected animals. Comparative studies using trypanosusceptible (Boran) and trypanotolerant (N'Dama) cattle revealed both qualitative and quantitative differences in these T-cell responses between the two breeds of cattle. The T-cell response was not only observed earlier post-infection in N'Dama cattle, but the cells showed a higher degree of trypanosome antigen-specific proliferation *in vitro*, and the response was maintained throughout the period of study. In contrast, the response observed in Boran cattle was transient and the degree of *in vitro* antigen-specific T-cell proliferation lower.

In an attempt to elucidate which subsets of T cells were involved in this trypanosome antigen-specific T-cell proliferation, the response of individual subpopulations of CD4+, CD8+ and γδ T cells to purified trypanosome antigens was studied in one Boran and one N'Dama during primary infection with *T. congolense*. These initial studies have now been repeated in a further two N'Dama and one Boran. The result of these studies showed that all T-cell subpopulations from both breeds of cattle exhibited negligible proliferation when stimulated with soluble VSG antigen. This result confirmed our previous studies with nitrocellulose bound antigens suggesting that the anti-VSG antibody response was T-cell independent. The situation with the other trypanosome antigens was quite different. Cysteine protease stimulated both CD8+ and γδ T cells from Boran to proliferate slightly. However, treatment with a trypanocidal drug was still required. Following treatment a response was observed involving

CD4+, CD8+ and γδ T cells; however, this response was very short-lived. By contrast, the N'Dama exhibited a marked proliferation of both CD8+ and γδ T cells when stimulated *in vitro* with this antigen. CD8+ T cells appeared to reach the peak of their response before the γδ cells which peaked later, possibly suggesting a regulatory role for the γδ T cells. The response was maintained throughout the period of study with high levels of stimulation. Trypanosome proteins between 100 and 140 kDa M.W. (100 kDa antigen complex) again stimulated CD8+ cells from Boran to proliferate slightly. These proteins stimulated predominately γδ T cells in N'Dama, although a response was also observed in CD8+ cells to a lesser extent. These responses were observed from 28 days post-challenge compared to those observed to the cysteine protease which were observed as early as 14 days post-challenge.

TR3.13 γδ T-cell function in N'Dama cattle

Scientist: J.N. Flynn
Technician: R. Saya

Preliminary studies on the activation of T-cell subsets from the peripheral blood of N'Dama and Boran cattle during primary tsetse-transmitted infection with *T. congolense* suggest that γδ T cells may be involved in the superior ability of N'Dama cattle to resist the effects of infection with trypanosomes. Following infection, CD8+ and γδ T cells from an N'Dama exhibited marked *in vitro* proliferation when stimulated with the 33 kDa trypanosomal cysteine protease. Likewise a 100 kDa antigen complex stimulated predominately γδ T cells in N'Dama, although a response was also observed in CD8+ T cells to a lesser extent. In contrast, the cysteine protease caused only slight proliferation of CD8+ and γδ T cells from a Boran. The 100 kDa complex also stimulated minor proliferation of CD8+ T cells from this animal.

Four T-cell lines were established from the N'Dama. Phenotypic analysis of these lines demonstrated that one was comprised entirely of CD8+ T cells, whilst the other 3 lines comprised between 47 to 95% γδ (WC1) T cells, the remainder being the CD8+ cells. These latter γδ T-cell lines also expressed CD3 and a proportion of the cells expressed CD2. Using two monoclonal antibodies raised against the subsets of the γδ TCR, one line expressed neither of these antigens whereas the other two expressed the antigen recognized by CACT B6A exclusively. Antigen recognition studies have shown that these γδ T-cell lines are specific for the 100 kDa complex and one of them responds on MHC mis-matched antigen presenting cells suggesting that the response of the γδ cells is not MHC-restricted.

Cloning of these lines has not been successful so far. One cell line has been transformed with *T. parva* which should improve cloning efficiency and also provide large numbers of cells for analysis.

TR3.14 B-cell responses in trypanosome-infected cattle

Scientists: J. Naessens, D. Williams
Technicians: J. Nthale, M. Opollo
Student: J. Busa

CD5+ B cells in cattle are present in peripheral blood and spleen, but not in lymph nodes, tonsils or Peyer's patches. Compared to classical B cells, they express normal levels of B-cell markers, but have higher levels of surface IgM. We failed to find evidence for IgD on bovine B lymphocytes. The CD5+ B cells expressed CD11b (Mac-1). A small population was also identified that carried CD11b but not CD5. In cattle infected with *Trypanosoma congolense*, a dramatic increase in the percentage of CD5+ B cells in blood and spleen was observed. This increase occurred 7–10 days after the first parasites were detected in the blood and correlated with the increase in serum IgM and the increase in the absolute number of B cells that are normally found in trypanosome-infected animals. The increase in B cells was found to be due solely to the expansion of the CD5+ B-cell subpopulation.

TR3.15 Characterization of variant-specific antibody responses and the rate of VAT turnover in trypanotolerant and susceptible breeds of cattle infected with *T. congolense*

Scientist: D.J.L. Williams
Research Associate: K. Taylor
Technicians: F. McOdimba, M. Opollo

Previous results indicated that N'Dama cattle had higher titres of antibody specific for populations of parasites isolated from peripheral blood following a *T. congolense* infection than did Borans. It was not apparent from these studies whether the N'Damas produced higher levels of antibody to the same variants as the Borans, or whether they recognized a greater range of the the variable antigenic type (VAT) repertoire than Borans. In order to address this question a panel of clones was produced from stabilates taken from 3 N'Damas and 3 Borans during the first wave of parasitaemia (day 12) and on days 17, 21, 28 and 35 post-infection. In this way 30 clones were selected. A panel of clone-specific antisera was raised and used to characterize these VATs. Using FACS analysis it was shown that some clones (four) were isolated from several animals. However 14 antigenically different clones were identified; of these 6 were from N'Damas and 8 from Borans, and for each time point there was at least one clone from an N'Dama and one from a Boran. These clones are now being used as target antigens in an ELISA to measure VSG-specific antibody present in the sera of infected N'Dama and Borans.

TR3.16 Studies on the antibody-induced directional movement of VSG molecules on the trypanosome surface

Student: D. Russo
Supervisors: D. Grab, D. Williams

Anti-VSG Fab fragments specifically bound to the surface of *Trypanosoma brucei brucei* S427 cl.22. After incubation on ice with a sub-saturating dose of antibodies, the trypanosome surface was diffusely labelled as visualized using an FITC-conjugated anti-species-specific antibody added after trypanosome fixation. After 2.5 hours of incubation at 37 °C, the whole length of the flagellum was strongly labelled by the Fab fragments, but fluorescence from the rest of the cell body had disappeared. After a further 2.5 hours at 37 °C labelling was no longer detectable on the trypanosome surface. Low amounts of anti-VSG Fab fragments were detected intracellularly after their clearance from the surface (5 hours at 37 °C) using electron microscopy or immunofluorescence on permeabilized trypanosomes. However, if a cocktail of protease inhibitors was added to the suspension, Fab fragments could be detected in large amounts within a single fluorescent vacuole, situated between the nucleus and flagellar pocket of the trypanosomes. The movement of the Fab-VSG complex was inhibited at temperatures below 4 °C and when the medium contained 2-deoxy-D-glucose in lieu of D-glucose. In addition, it was immediately and reversibly inhibited by increasing the NaCl concentration in the medium to 200 mM. When a critical level of surface-bound antibody was reached, the process was also inhibited by protein synthesis inhibitors, protease inhibitors and temperatures below 20 °C. In contrast, when the trypanosome surface was biotinylated on ice, no clearance of the VSG-biotin was observed during 4 hours of incubation at 37 °C as visualized using FITC-conjugated streptavidin, added after trypanosome fixation.

TR3.17 *T. congolense* cysteine protease—its identification and specific antibody responses in infected cattle

Scientists: E. Authié, D.J.L. Williams
Research associate: C. Taylor
Technicians: D.K. Muteti, B.G. Muchiri

An antibody-ELISA using the *T. congolense* cysteine protease purified by monoclonal (MAb) affinity-chromatography was developed. Specific antibody responses during a primary challenge with *T. congolense* were compared in 70 cattle belonging to different breeds (N'Dama, Boran, N'Dama × Boran, Baoule, West African Zebu, Friesian). There was a positive correlation between the titre of anti-protease antibodies (IgG) 40 days post-infection and the degree of resistance to trypanosomiasis of each group of cattle. Thus, the association between an IgG response to the cysteine protease and resistance to trypanosomiasis was confirmed.

In N'Dama and Baoule cattle, anti-protease antibodies of the IgG_1 class were generally detected by ELISA 15 to 30 days post-infection. No free anti-protease IgM was detected, confirming previous results from Western blot analysis. However, using

a 'sandwich'-ELISA, IgM-immune complexes were detected in the plasma of all animals from day 15 post-infection. Specific IgM is thus elicited in both breeds, and all of it appears to be incorporated into immune complexes. Although IgG-immune complexes were not detected in a similar assay, it is possible that they are also produced but are rapidly removed from circulation. In an antigen-capture ELISA using both anti-protease MAb and a rabbit polyclonal antibody, circulating antigen was detected in the plasma of all infected animals from the onset of parasitaemia. The circulating antigen was isolated from fresh plasma of infected Friesians, 20 days after infection, using the MAb-affinity column. The 33 kDa antigen and high molecular weight complexes involving predominantly IgM were identified by subsequent SDS-PAGE and immunoblotting. The mixture displayed proteolytic activity on Azocoll and this activity was inhibited by E-64, a specific inhibitor of cysteine proteases. Thus, the protease appears to be present as an active enzyme in the plasma of infected animals from early infection.

Host proteins such as the heavy chain of IgGs and the alpha chain of complement factor C3 were cleaved *in vitro* by the trypanosome enzyme. Possible expression of proteolytic activity *in vivo* and its relevance to pathology are being studied.

TR3.18 The 69 kDa immunodominant invariant antigen of trypanosomes

Scientists: E. Authié, A. Boulange, N. Murphy, D. Nandan, T. Pearson*, M. Shaw, D. Williams, T. Baltz†
Research Associate: C. Wells
Technician: D.K. Muteti, B.G. Muchiri

*Department of Biochemistry and Microbiology, University of Victoria, BC, Canada
†Laboratoire de Biologie et Immunologie Parasitaires, Universite de Bordeaux II, France

Monoclonal (MAb) and polyclonal (PAb) antibodies against the 69 kDa immunodominant antigen of trypanosomes have been produced. MAb 1F3 and rabbit PAb have been used to localize the antigen by immunofluorescence and immunoelectron microscopy on paraformaldehyde-fixed parasites. The antigen detected by the rabbit PAb is present in the endoplasmic reticulum, along the nuclear membrane, in lysosome-like organelles and in the flagellar pocket. The epitope recognized by MAb 1F3 is detected in the perinuclear area and in the endoplasmic reticulum. This localization gives further indication that the 69 kDa antigen may be a member of the HSP70 family.

A *T. congolense* genomic library in lambda-gt 11 was screened using an infected bovine serum. This serum was made monospecific by absorption on a trypanosome lysate depleted of 69 kDa protein. Ten genomic DNA clones were detected. Their DNA inserts cross hybridized; however, the fusion proteins induced on plates were not recognized by the other available antibodies. The DNA insert from one of the genomic recombinant clones is being sequenced. In addition, a *T. congolense* epimatigoste cDNA library was screened using a monospecific rabbit PAb. Seven clones

were detected. Their DNA inserts cross hybridized, and also hybridized with the DNA of the genomic clones that had been detected by bovine antibodies.

TR3.19 Experimental infection with an haemorrhage-causing stock of *Trypanosoma vivax* in N'Dama and Boran cattle

Scientists: D.J.L. Williams, L. Logan-Henfrey, E. Authié, S.K. Moloo
Visiting Student: C. Seely*
Technicians: J.M. Kabata, F. McOdimba, M. Opollo.

*Zoology Department, University of Glasgow, Scotland

Four male N'Dama *(Bos taurus)* and four age-matched male Boran *(Bos indicus)* cattle were infected by *Glossina morsitans centralis* with a *T. vivax* stock isolated from Galana, Kenya. In Ayrshire cattle, this stock is known to cause a severe haemorrhagic disease. All eight cattle became parasitaemic 8 to 9 days after infection. Peak parasitaemia was reached 15–16 days after infection. The number of parasites in the blood declined from 24 days after infection onwards. Packed red cell volumes (PCV) decreased from 7 days after infection. A lowest mean PCV of 24.2% was reached on day 25 after infection in the Borans and of 16.75% on day 26 in the N'Damas. One of the four N'Damas showed a PCV of 13% on day 26 after infection and it was treated with 7 mg kg^{-1} diminazene aceturate. The other 7 cattle recovered without requiring treatment.

Thrombocytopaenia was observed in both groups of cattle between days 14 and 21 after infection and the counts remained below preinfection values until the end of the experiment (day 56). There was a decrease in the leukocyte count in both groups up to day 14 after infection. Thereafter the leukocyte count increased and a leukocytosis was observed in the N'Damas on day 27 and in the Borans on day 34 after infection. The alterations in the leukocyte count were attributable to changes in the lymphocyte count. A neutrophilia was observed on days 7, 14 and 48 in the Borans and on day 55 following infection in the N'Damas.

Antibody responses to invariant trypanosome antigens were analysed. An immunodominant 69 kDa antigen was identified which elicited an IgM and IgG response in both groups of cattle. This antigen was found to be identical to a 69 kDa immunodominant antigen of *T. congolense*. The majority of the antigen-specific antibodies were of the IgG_1 class, but those of the IgG_2 class were also detected. No differences were observed between the groups in the pattern of recognition nor the isotype elicited. Antibody bound to the surface of erythrocytes was occasionally detected. No anti-platelet activity was observed.

In conclusion, although N'Dama cattle have been shown to control infections with both *T. congolense* and *T. vivax* better than Boran cattle, there was no difference between the two breeds in their ability to control an infection with a *T. vivax* stock associated with haemorrhagic disease.

TR3.20 Comparative macrophage erythrophagocytosis and monocyte surface membrane antigen expression during *Trypanosoma vivax* IL 2337 infection in Boran and N'Dama cattle.

Student: V.O. Taiwo
Supervisors: D.J.L. Williams, L. Logan-Henfrey

Erythrophagocytosis is thought to be a major cause of trypanosome-induced anaemia in cattle. In order to analyse the mechanisms of erythrophagocytosis, an *in vitro* assay was established in which peripheral blood monocytes were purified and incubated with ^{51}Cr-labelled red blood cells. The release of ^{51}Cr was used as a measure of erythrophagocytosis.

Four N'Dama (trypanotolerant) and four Boran (susceptible) cattle were infected with *Trypanosoma vivax* IL 2337 by *Glossina morsitans centralis*. This *T. vivax* stock is known to cause acute haemorrhagic disease in Ayrshire cattle. There was no difference between the two groups of cattle in the prepatent period or in the level of parasitaemia. The packed cell volumes (PCV) decreased from 7 days post-infection. The lowest PCVs were detected on day 26 in the N'Damas (16.75%), and on day 25 in the Borans (24.2%) and thereafter increased.

Erythrophagocytosis was measured in three of the N'Damas and three of the Borans using the ^{51}Cr release assay. Erythrophagocytosis increased in both groups of cattle following infection and there was no significant difference between the two groups in the level of erythrophagocytosis.

Flow cytometry was used to measure surface expression of antigens recognized by monoclonal antibodies IL-A15 (CD11b), IL-A21 (MHC-class II), IL-A24 and IL-A109. Large inter-animal variations were observed in the number of cells expressing the antigens recognized by this panel of MAbs. No significant alterations in their expression were observed during infection.

The N'Dama and Boran cattle in this experiment were equally susceptible to an infection with *T. vivax* IL 2337. The levels of erythrophagocytosis increased in both groups following infection; however no significant differences in either the anaemia or in levels of erythrophagocytosis *in vitro* were detected between the two groups.

TR3.21 Identification of bovine progenitor cells

Scientists: J. Naessens, L. Logan-Henfrey, S. Kemp
Technicians: P.M.J. Kamau

Attempts have been made to obtain monoclonal antibodies (MAbs) to erythroid progenitor cells. As a source of progenitor cells, FACS-sorted bone marrow cells that were 'lineage' negative (negative for a number of mature lymphocyte markers) and highly positive for MAb VC15 were used. This cell population contained about 70–80% progenitor cells, most of which (90%) were erythroid progenitors. Three fusions were performed, but no antibodies to cells from the erythroid lineage were obtained. Only MAbs against myeloid cells were produced. To improve the likelihood of ob-

taining MAbs specific for cells of the erythroid lineage, mice have been immunized with transferrin-receptor positive bone marrow cells.

Measurement of spleen colony forming units (CFU-S) provides an easy way of monitoring mouse stem cells. We have tried to measure bovine colony forming units in spleen from different irradiated mouse strains. Bovine bone marrow cells were transferred to irradiated mice, and after 12 days the spleens were examined for nodules. Nodules were occasionally observed in irradiated Balb/C mice, but were consistently present in irradiated SCID/beige mice. Bovine-specific DNA-sequences were found in spleens with nodules, indicating that the nodules were of bovine origin.

TR3.22 The role of dyshaemopoiesis in bovine trypanosomiasis

Scientists: L. Logan-Henfrey, A. Andrianarivo, J. Naessens, V. Anosa*
Research Associates: L. Gaidulis, C. Wells
Technicians: P. Muiya, F. McOdimba, J. Kamau

*Department of Veterinary Pathology, University of Ibadan, Nigeria

Techniques have been developed to collect sequential bone marrow samples from adult cattle and the standardization of the short-term bovine bone marrow culture assay. Conditioned media collected from leukocytes stimulated with lectins such as Concanavalin A (Con A) and phytohemagglutinin (PHA) were previously used in short-term culture assays. We have found Con A and PHA to be highly suppressive to BFU-E (Burst-forming units-erythroid), the earliest erythroid progenitor we can detect in bovine bone marrow cultures. Sequential bone marrow biopsies allow the same animals to be followed throughout a trypanosome infection. A group of 5 adult Borans which have previously been challenged with *T. congolense* IL-13-E3 and treated were reinfected. Peripheral blood haematology, cytology, histology, EM, FACS analysis of the bone marrow, and bone marrow and blood culture were all carried out. Sera and plasma were collected from the blood, and plasma from the bone marrow. Cytospins were made of bone marrow cells and stored at –70 °C for future *in situ* hybridization studies.

Blood cultures were followed for a group of *T. vivax*-infected Boran and N'Dama cattle. As the animals recovered BFU-E, CFU-E (colony forming unit-erythroid) and CFU-GM (granulocyte, macrophage) were detected in the blood. Blood and bone marrow were collected from a group of chronically infected Friesian cattle (12 weeks and at 15 weeks). Very few BFU-E were detected in the bone marrow and none in the blood.

TR3.23 Influence of bone marrow-derived cell populations on trypanotolerance phenotype

Scientists: A.J. Teale, S. Kemp, J. Naessens, D. Kennedy
Research Associate: S. Leak

One pair of bovine bone marrow chimaeras has been produced by implanting an N'Dama embryo and a Boran embryo into a Boran dam. The two calves were born in December 1990. Analysis of polymorphic CD5 markers (IL-A67—*B. indicus;* CC17—*B. taurus)* suggested that the animals were chimaeric at the T- and B-cell level but that the ratio of *indicus:taurus* is changing over time. Microsatellite analysis and MHC typing confirmed that these animals are chimaeric.

TR3.24 Neuroendocrine dysfunction during bovine trypanosome infections

Student: G. Abebe
Scientists: R. Eley*, O.K. oleMoiYoi, L. Logan-Henfrey, M. Shaw
Supervisors: R. Eley*, O.K. oleMoiYoi

*Institute of Primate Research, National Museums of Kenya, Karen, Kenya

Studies of the hypothalamic-pituitary-adrenal (HPA) axis in *T. congolense*-infected Boran cattle suggest that the low responsiveness of the HPA axis to bovine corticotrophin releasing factor (bCRF) and insulin during trypanosome infection is due to an impaired pituitary response. Basal cortisol, ACTH and thyroxine (T4) levels were lower in trypanosome-infected than control animals. Likewise, a significant difference in mean concentration of both ACTH and cortisol was noted between trypanosome-infected and uninfected cattle stimulated with exogenous bCRF. The dysfunction of the pituitary could be mediated directly by trypanosome metabolites or indirectly by cytokine mediators such as TNF. Activated macrophages have been identified in pituitary blood vessels. Cytokine excretion in this micro-environment may have an adverse effect on pituitary function. IL-1 and IL-6 are known to activate the HPA axis, and TNF inhibits the secretion of some pituitary hormones. In a small pilot study in which two trypanosome-infected Boran cattle were examined 56 days post-infection, a second lesion has been identified in the adenohypophysis in somatotrophic cells. Thus, trypanosome-induced early hypothalamic-pituitary lesions could lead to secondary dysfunction in target endocrine and non-endocrine tissues due to low levels or lack of pituitary-tropic hormones.

EPIDEMIOLOGY AND SOCIOECONOMICS

ESE1 Epidemiology of livestock disease control

Scientists: B.D. Perry, J.C. Barrett*, J.J. Curry, S.K. Hargreaves*, S. Maloo†, S.K. Mining†, S.P. Morzaria, W. Thorpe§, U. Ushewokunze-Obatolu*, A.S. Young
Research Associate: R.L. Kruska

*Department of Veterinary Services, Harare, Zimbabwe
†Kenya Agricultural Research Institute (KARI), Muguga, Kenya
§International Livestock Centre for Africa (ILCA), Mombasa, Kenya

Effective and sustainable disease control programmes should be based on reliable data as to the effect of the diseases to be controlled on livestock production in an area. ILRAD is one of the key institutions in the development of sensitive and specific diagnostic tests for the identification of tick-borne disease parasites, antigens and antibodies to them. The challenge is to use these tools on specimens taken from a representative sample of the livestock populations of an area and the production systems in which they are kept. The Socioeconomics Programme is seeking to develop quantitative sampling strategies and assists national programmes in the analysis and presentation of results. Furthermore, data so produced will be of value in the development of models to predict the occurrence and dynamics of disease under situations where detailed data are not available.

Stratified serological studies of theileriosis and other tick-borne diseases have now been completed in two areas of Kenya—Kaloleni Division of Coast Province and Uasin Gishu District of Rift Valley Province. In addition, the Department of Veterinary Services in Zimbabwe has carried out a national stratified random sample survey of cattle.

A sentinel calf study has been completed in Kaloleni Division to determine age-specific incidence and case-fatality of East Coast fever (ECF) under natural challenge, and a study to evaluate the immunity of naturally infected cattle to the designated immunizing stock of *T. parva* is under way.

Results in Kaloleni Division have shown a generally high prevalence of schizont antibodies to *T. parva* in all strata (74%), suggesting a high level of population immunity. In the face of reports of clinical ECF in the area, this suggests that in general terms the cattle population largely comprises ECF survivors. The high prevalence of antibodies in agroecological zone coastal lowlands (CL)3, in both taurine-Zebu cross and Zebu cattle of all age groups, including those under 18 months of age, suggests that infection, and thus mortality, occurs early in life. There was a lower level of antibody prevalence recorded in animals over 3 years old kept under zero grazing management; this could be a result of effective tick control under zero grazing on some premises, and of the introduction of adult stock from breeding farms free of ECF. With regard to other tick-borne diseases, there was a lower prevalence of antibodies to *B. bigemina* (42% c.i. 2–59) and *A. marginale* (26%, c.i. 12–43) in taurine-Zebu cross in CL3 than in other agroecological zones.

In Uasin Gishu, a much lower overall prevalence to *T. parva* was detected (27–34%), and there was considerably less variation by agroecological zone, cattle type and management system than in Kaloleni. Variations in antibody prevalence were detected between cattle whose watering source was on-farm (17–25%) and those whose

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was off-farm (37–47%). There was a slightly lower prevalence in those who reportedly dip their cattle (21–29%) compared to those who spray (31–48%) or who dip and spray (31–45%). Highest antibody prevalence rates were seen in Moiben Division (37–47%) and lowest in Ainabkoi (11–25%). Higher prevalence rates were recorded in small and medium (1–99 acre) size farms (32–42%) than in large farms (16–26%).

In the sub-humid coastal region of Kenya, economic and epidemiological evidence indicates a justification for immunization of taurine-Zebu cross dairy cattle in zone CL3. Economic justification for the immunization of Zebu calves in the drier CL5 zone appears probable, and further studies to define losses due to ECF in these animals are under way. In Uasin Gishu District, a broader target population for immunization appears to exist, but farm level economic studies, due to start shortly, may better define this.

ESE2 Social, economic and environmental characterization of target farming systems for livestock disease control

Scientists: J.J. Curry, B.D. Perry, A.W. Mukhebi, R.A. Huss-Ashmore*

*University of Pennsylvania, Philadelphia, PA, USA

An improved understanding of the socioeconomic context of livestock disease and its control can be gained by analysing the linkages between the farming unit and the livestock disease control system as mediated by farm/livestock management. For resource-limited, small-scale farmers in Africa, these linkages are at present poorly specified and understood. Since 1989, collaboration with the Kenya Agricultural Research Institute (KARI) and the International Livestock Centre for Africa (ILCA) has been carried out to examine these linkages by adopting a research strategy similar to that of farming systems research (FSR). This approach to livestock disease control is unique, since most farming systems research has been carried out in order to improve crop production. Central to both FSR and ILRAD's Socioeconomic Programme approach is the necessity of understanding farmers' circumstances through diagnostic surveys and farm characterization exercises which facilitate the identification of target groups of farmers for technology development and extension.

Two farm characterization studies have been carried out to date in collaboration with KARI and ILCA at two sites in Kenya—Kilifi District, Coast Province, and Uasin Gishu District, Rift Valley Province—in order to characterize target populations for pilot immunization against East Coast fever (ECF) using the infection and treatment method. At the Kenya Coast, livestock production systems can be classified according to agro-ecological zone, cattle type and grazing system. As previously reported, grade cattle kept under zero grazing conditions in coastal lowland zones (CL)3 and CL4 were found to be more productive and more susceptible to risk of ECF infection than were Zebu cattle kept under less intensive feeding regimes.

A multi-stage farm characterization exercise has been carried out in Uasin Gishu District. During Phase I and II interviews, agroecological zone and cattle type were found to be less important for characterizing farming systems than was the case in Kilifi. Instead, farm size was found to be an important discriminating variable and was

closely associated with cattle herd size and livestock management practices. In a farm-level pilot survey, large-scale farms (>100 acres) in the sample (N=71) were significantly different from medium (11–99 acres) and small (1–10 acres) farms on nearly all production and consumption variables (see abstract ESE4). There were, however, no significant differences between the small and medium-scale farm categories in the sample. This implies more work will need to be done, particularly with regard to the medium-scale category, in order to specify target populations prior to immunization in Uasin Gishu.

ESE3 Financial and economic impacts of alternative livestock disease control strategies

Scientists: A.W. Mukhebi, B.D. Perry and W. Thorpe*
Research Associates: R. Kruska, E. Mussukuya*
Technicians: M.S. Baya, E. Rugema*

*International Livestock Centre for Africa, Mtwapa, Kenya

The objective of this project is to assess and predict the financial and economic impacts of alternative livestock disease control strategies under different livestock production circumstances. In 1991 the project sought (a) to characterize the farm household economy and assess the contribution of livestock to the household economy in Kaloleni Division of Kilifi District, Coast Province of Kenya, an area targeted by the Kenya Agricultural Research Institute (KARI) for pilot immunization of cattle against East Coast fever (ECF) and (b) to use some of the information generated from the farm household study as well as information from the pilot immunization to provide a preliminary assessment of the financial impacts of ECF immunization in Kaloleni Division.

(a) The farm household economy in Kaloleni Division. A survey of 77 farm households in Kaloleni Division was conducted during 1990 and its preliminary results were reported in 1990. Further analysis of the survey data was done during 1991 and the substantive results may be summarized as follows. Households with cattle owned greater resources of land, labour and capital than those without cattle, and those with grade (improved taurine-Zebu cross) cattle had larger amounts of land and capital than those with Zebu cattle. Consequently, households with cattle generated higher (total as well as per capita) farm and household incomes than those without cattle, and those with grade cattle achieved greater incomes as well as higher resource productivity than those with Zebu cattle. Thus, greater household income was positively associated with the ownership of cattle, and particularly grade cattle, which was in turn positively associated with access to greater resources. In general, livestock contributed 70% of farm investments, 40% of farm (crop and livestock) income and 12% of household (farm and off-farm) income. The most important constraints to livestock production were reported to be cash (for the purchase of breeding stock or inputs), diseases, inadequate feed (particularly during dry periods) and inadequate labour (for herding or for forage cut-and-carry) in that order of importance. On the

average of the whole household sample, off-farm income constituted 69% of total household income.

The farm household economy of Kaloleni Division can be characterized as a smallholder, resource-poor, subsistence-oriented economy. It is based upon small land holdings often fragmented into two or more parcels or farms. It employs a low-input and low-output level of technology—the use of traditional farm-produced inputs dominates; there is limited use of purchased high yielding inputs and crop varieties; production is characterized by low crop and livestock yields; farm-generated income is low, and there is considerable reliance upon off-farm income. Women play a dominant role, both as farm managers and principal suppliers of household family labour.

If the disease risk could be lowered through improved, low-cost, financially profitable and acceptable control methods, it is likely that more farmers in Kaloleni Division could be encouraged to invest in grade cattle production, especially if concomitant constraints (e.g, cash and nutrition) were also addressed to allow maximum benefits from improved livestock disease control to accrue to farmers.

(b) A spreadsheet model (see abstract ESE7) was applied to assess the financial implications of immunization of grade cattle against ECF in the whole of Kaloleni Division. The total population of grade cattle in the division was estimated to be about 900. The preliminary analysis was made using cattle production data from the farm household survey, data on the costs of field delivery of immunization from the pilot immunization conducted in the division during November 1990 by KARI, as well as various assumptions regarding the occurrence of ECF in the division and the likely effects of immunization on grade cattle production in households currently keeping grade cattle.

The preliminary results include an estimated economic loss caused by ECF in the division, the cost of the pilot immunization per cattle head, the break-even cost of immunization per head, and the estimated increase in net income generated from cattle, under various assumptions of reduction in the frequency of acaricidal application following immunization. The results will be refined using more accurate epidemiological data presently being generated from the post-pilot immunization monitoring of cattle performance.

Methodologies developed for studying the impacts of controlling tick-borne diseases will be applicable, with appropriate modifications, for assessing the impacts of trypanosomiasis control and indeed the control of other livestock diseases.

ESE4 Nutritional impact of livestock disease control

Scientists: R.A. Huss-Ashmore*, J.J. Curry, A.W. Mukhebi

*University of Pennsylvania, Philadelphia, PA, USA

Studies have examined ways of including nutritional concerns within its programme of socioeconomic research. This has involved (1) the development of a conceptual framework for linking livestock interventions to household nutrition, (2) identification of appropriate methods for measuring nutritional impact, and (3) pilot application of these concepts and methods to research areas in Coast Province and Uasin Gishu

District. Previous nutrition studies conducted at the Kenya Coast were reviewed and appropriate research topics and data collection methods identified for further research by the KARI/ILCA/ILRAD research group in Kaloleni Division. A number of points of articulation between the livestock system and the nutritional system of the farm household have been identified. These include income, resource allocation (e.g. crops versus livestock), food consumption and human disease risk.

Data on several of these linkages were collected during a pilot survey of farm characteristics and food consumption patterns using a sample of 72 households drawn from the Uasin Gishu serology study. There was considerable variation on a number of production variables between large-scale farms (>100 acres), medium (11–99 acres) and small-scale farms (<10 acres). On average, large-scale farms in the sample produce 18 times as much milk and reserved 4.5 times as much for household use as do small-scale farmers. Average estimated daily incomes derived from milk ranged from Kshs. 10.15 for small farms to Kshs. 344.40 for large farms. Household milk consumption was significantly different between households with large farms (2.2 ltr/CU/day) and either medium farms (1.6 ltr/CU/Day) or small farms (1.0 ltr/CU/day).

ESE5 Delivery, adoptability and sustainability of animal health interventions

Scientists: J.J. Curry, B.D. Perry, A.W. Mukhebi
Research Associate: R.L. Kruska
Student: D.G. Onchoke*
Supervisor: M. Kyule*

*University of Nairobi School of Veterinary Medicine, Kabete, Kenya

The identification of issues of delivery, adoptability and sustainability of animal health interventions has been carried out in two principal subject areas. Studies of local knowledge of livestock disease and disease risk have been conducted at both research sites in Kenya (see abstracts ESE1–4). As previously reported, ethnoveterinary studies conducted in 1989 in Kilifi District indicate that farmers at the Kenya coast use four major theileriosis descriptors, the most common of which is applied restrictively to theileriosis in calves. The study found, in general, poor understanding of the role of ticks in theileriosis transmission. In 1990, a similar study was carrried out in Uasin Gishu District, Rift Valley Province. Unlike Kilifi, the terms used in Uasin Gishu which most often describe clinical signs associated with theileriosis are applied to all age classes of cattle. There also appears to be a better understanding of the role of ticks in disease transmission by Uasin Gishu farmers, as indicated by their frequent use of the Kalenjin term for tick *(kerebesiet)* as a descriptor for clinical signs associated with ECF. Lexicons of over 100 descriptors for disease syndromes in Swahili, English and local languages have been constructed for each area. These should assist the Kenya Agricultural Research Institute (KARI) in implementing pilot immunization against ECF at the local level.

Spatial and economic techniques have been employed in collaboration with national programmes in Kenya and Zimbabwe to evaluate the infrastructure necessary

for delivery of disease control interventions. In Zimbabwe, national coverage of livestock disease control infrastructure is being examined using GIS to analyze the distribution of animal health centres and dip tanks (see abstract ESE6 for description). Collaborating scientists have conducted economic analyses of various tick control strategies for Zimbabwe. A study of current dipping percentages and frequency has been carried out by scientists in Zimbabwe, in collaboration with ILRAD, for each dip tank in the country. In Kenya, working with researchers at KARI, the cost of a pilot immunization campaign in Kilifi District has been estimated.

ESE6 Development of geographic information systems and spatial databases for the study of the impact of livestock disease control

Research Associate: R.L. Kruska
Scientist: B.D. Perry
Collaborating Scientists: H. Croze*, U. Ushewokunze-Obatolu†, G. Mullins, W. Thorpe§, J. Scott
Technician: M.S. Baya

*United Nations Environment Programme, Nairobi, Kenya
†Department of Veterinary Services, Harare, Zimbabwe
§International Livestock Centre for Africa, Mombasa, Kenya

ILRAD has acquired and integrated three Geographic Information Systems (GIS). 1) GRASS GIS: a raster-based system running on two SUN Graphics Workstations under UNIX, 2) pcARC/INFO: a vector-based system running on two IBM PCs (on loan from the UN Environment Programme's (UNEP) Global Resource Information Database (GRID), and, 3) IDRISI: a raster-based system running on an IBM PC.

All entry of spatial data (digitizing) is accomplished with pcARC/INFO, because of its wide use and acceptance as a standard format. This makes the databases developed easily transferrable to other institutions such as GRID. This data format is also the easiest to convert into other GIS formats. Recently, the two SUN Workstations have been networked and can share both programme and data resources.

Database development at the continental level has continued and will supplement databases previously compiled in collaboration with the GRID. Continental distributions of wildlife hosts of selected animal diseases have been digitized, including waterbuck and eland, from the best available sources. Also, reported distributions of tick vectors *Amblyomma variegatum* and *Amblyomma hebraeum* have been compiled and added to the database. Murdock's 1957 continental map of African ethnic groups has been digitized and will be supplemented with more up-to-date anthropological information.

Regional and Kenya Divisional level database development is under way in collaboration with the International Livestock Centre for Africa (ILCA) and the Kenya Agricultural Research Institute (KARI). A large amount of information has been added to the GIS including cattle census, serological surveys, agro-ecological zones, dairy marketing survey farms and extensive transportation networks. Agro-ecological

zones for the sub-humid coastal zone of eastern Africa have also been prepared for projection of the Kaloleni research findings to the whole region.

The Zimbabwe country-level GIS database continues to be developed in collaboration with the Department of Veterinary Services (DVS), Zimbabwe. This extensive database of over 60 layers of information is currently being supplemented with a new communal land dip tank survey just completed by DVS. In addition, verification of the 2,500 dip tank locations is currently being undertaken by DVS/SEP. Also, high resolution normalized difference vegetation index (NDVI) satellite data is now being collected for the whole country for future evaluation of tick habitat. Finally, a survey of certain species of wildlife (natural hosts of vector-borne diseases) has been initiated by SEP/DVS to map current distributions.

ESE7 Development of models for assessing the impact of livestock disease control

Scientists: B.D. Perry, A.W. Mukhebi, T.T. Dolan, S.P. Morzaria, A.J. Teale, A.S. Young
Collaborators: J.R. Richardson*, J. McMahon†, G.F. Medley§, G. Gettinby‡, R.A.I. Norval¶
Research Associate: R.L. Kruska

*Texas Agricultural Experiment Station, Texas A & M University, USA
†Australian National University, Canberra, Australia
§Imperial College, London, UK
‡University of Strathclyde, UK
¶University of Florida, Gainsville, USA

Following previous use of the model CLIMEX to predict the distribution of *R. appendiculatus* in Africa, a study was set up to quantitatively validate CLIMEX when used on a high-resolution (5 minute) interpolated climate database for Zimbabwe.

The distribution of ecoclimatic index (EI) values were evaluated by comparison with reported tick and disease occurrence data in a GIS. Furthermore, correlations of predicted suitability were carried out with natural regions (a rainfall-driven classification from I, with the highest rainfall to V, the driest region) and land-use (e.g. commercial, communal) categories. Good correlations were detected between the presence of an EI value of one or greater and the recorded occurrence of *R. appendiculatus*. Correlations with theileriosis outbreaks were less precise, but this was improved if known buffalo-associated outbreaks in the drier areas of the country in which *R. zambeziensis* is reported were not included in the analysis. Virtually all outbreaks were associated with commercial land use and natural regions I, IIa, IIb, and III.

The model CLIMEX, run on the high resolution interpolated climate database, was used to predict the distribution of the ticks *Boophilus decoloratus*, *B. microplus*, *Amblyomma hebraeum* and *A. variegatum* in Zimbabwe. There was a good correlation with predicted suitability and known *B. decoloratus* distribution, but poor correlation with *A. hebraeum*. The current distribution of *A. hebraeum* is closely associated with commercial land-use in natural region V. Although data on the distribution of wildlife hosts are still being assembled, initial results suggest that they are widely prevalent in

the commercial farming areas of regions IV and V, but not in either commercial or communal land-use areas of regions I–III. It is possible that the tick has been excluded from the higher rainfall areas, particularly the commercial lands, due to regular treatment of cattle with acaricides and the absence of alternative wildlife hosts there. However, this would suggest that reduction in the frequency of dipping in natural regions I–III, proposed as an alternative national strategy, could allow *A. hebraeum* to become established on cattle in the areas, and result in the reintroduction of heartwater.

A prototype computer spreadsheet model, developed in 1990 for estimating the economics of livestock disease control, was further refined in 1991. It is based on a defined (farm or above-farm) herd of cattle of a specified breed in a defined production system with a defined resource base. The parameters of the model include the size and structure of the cattle herd, the estimated impact of a disease in terms of incidence, case-morbidity and case-fatality, and the effect of a specified control method on the disease, and ultimately on cattle productivity and net income. Other financial or economic impact indicators such as marginal benefits, benefit-cost ratio and rate of return on capital can be calculated from the results of the model. Sensitivity analysis of important parameters such as disease incidence and mortality in calves can be undertaken. In addition, break-even analysis can be carried out to determine the break-even cost of the control method being evaluated, which is the maximum cost at which the control method would remain economically viable. Currently, the model runs deterministically for a study period of one year. However, the analysis can be repeated for each year for any desired planning horizon. A variety of disease control scenarios can be evaluated and the results compared.

The spreadsheet model was applied for preliminary estimates of the economic losses caused by East Coast fever (ECF) and the ex-ante economics of its control by the infection and treatment method of immunization in: a) the eleven countries in Africa where theileriosis (caused by *T. parva*) occurs, b) Kaloleni Division of Kilifi District, Coast Province of Kenya, and c) Uganda.

In order to improve ex-ante economic analysis and prediction of the likely impacts of improved control of livestock diseases, a general farm-level simulation model termed the Technology Impact Evaluation Simulator (TIES) is being developed in collaboration with the Texas Agricultural Experiment Station of Texas A & M University. TIES is a computerized whole farm simulation model designed to assist analysts in quantifying and predicting the financial, economic and nutritional impacts of alternative livestock disease control technologies. The model simulates the annual crop and livestock production, marketing, financial management, and household consumption aspects of a farm household over a 10-year planning horizon. The model uses a one-year time interval and simulates 10 years recursively, by starting each year with the ending debt and asset information for the previous year.

Evaluation of the financial, economic and nutritional impacts of alternative disease control technologies on a farm is a two-step process. First, TIES simulates the base farm (without new technology) for 10 years. In the second step, the model simulates the farm with the appropriate changes for the technology to be analysed. The results of the second step can then be compared with the base farm results. The key output variables projected by TIES include: Net Present Value, Benefit Cost Ratio, Internal Rate of Return, Average Annual Cash Receipts, Average Annual Expenses,

Average Annual Net Farm Income, Average Annual Household Consumption, and Present Value of Ending Net Worth. When fully developed, TIES will be amenable for the analysis and prediction of the farm-level impacts of controlling a variety of livestock diseases.

A prototype version of the model, together with a database management system, have been developed and verified. The verification was performed using farm-level data from selected farmers from Kaloleni Division in the coastal region and Uasin Gishu District in the highland region of Kenya, and discussion of preliminary results from the model with the selected farmers.

PUBLICATIONS

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Peregrine, A.S., Knowles, G., Ibitayo, A.I., Scott, J.R., Moloo, S.K. and Murphy, N.B. Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. *Parasitology* 102: 93–100 (905).

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STAFF PRESENTATIONS AT INTERNATIONAL MEETINGS

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R. Bishop

The Molecular Parasitology Meeting, Woods Hole, Massachussets, USA, September 1991. Paper presented: *A multicopy gene family in* Theileria parva. R. Bishop, B. Sohanpal, S. Morzaria, B. Allsopp, H. Baylis, E. Gobright and A. Musoke.

J. Buza

Tanzania Veterinary Association Scientific Conference, December, 1991. Arusha, Tanzania. Paper presented: *The erythropoietin responses in bovine trypanosomiasis.* J.J. Buza, L.L. Logan-Henfrey and D.J.L. Williams.

J. Curry

Workshop on the Socioeconomic Impact of Improved Tick and Tick-Borne Disease Control in Zimbabwe, Harare, Zimbabwe, May, 1991. Paper presented: *The human dimension of livestock disease control in small-scale farming systems.*

T. Dolan

Second EEC Meeting on Orientation and Coordination of Research on Tropical Theilerisosis, Anand, India, March, 1991. Paper presented: *Research on* Theileria parva *at the International Laboratory for Research on Animal Diseases.*

J. Doyle

BIOTASK/International Service for National Agricultural Research for the Consultative Group on International Agricultural Research, the Hague, Netherlands, September, 1991. Papers presented:

1. *Animal biosafety at ILRAD.*
2. *ILRAD's experience in patenting r-DNA vaccines.*

W. Fish

Gordon Conference on Bioenergetics, New Hampshire, USA, July, 1991. Paper presented: *Biochemical and molecular biological studies of the vacuolar ATPase of African trypanosomesi.* W.R. Fish, C.W. Muriuki, W.A.M. Kitungu, J.R. Young, M.D. Macklin and N.B. Murphy.

J. Flynn

11th European Immunology Meeting, Helsinki, Finland, June, 1991. Paper Presented: *Analysis of bovine T-cell responses during tsetse-transmitted trypanosomiasis.* J.N. Flynn and M. Sileghem.

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D. Grab

Federation of American Society for Experimental Biology 75th Annual Meeting, Atlanta, USA, April, 1991. Paper presented: *Tentative identification of an antigen associated with the surface of* Theileria parva-*infected lymphocytes*. D.J Grab, C.L. Baldwin, W.C. Brown and L. Innes.

A. Gray

Symposium on the Role of Animals in Feeding Africa, IDRC, Ottawa, Canada, September, 1991. Paper presented: *The role of International Laboratory for Research on Animal Diseases in realizing the potential of African livestock.*

Presentation to The Animal Diseases Research Institute (Agriculture Canada), Napean, Canada, September, 1991. Paper presented: *New technology for the control of haemoprotozoan diseases.*

H. Hirumi

VII Japan-German Cooperative Symposium on Protozoan Diseases, Berlin, September, 1991. Paper presented: *Present status of the* in vitro *cultivation of* Trypanosoma vivax. H. Hirumi and K. Hirumi.

44th Annual Conference, Japan Society Parasitology, Nagasaki, November 1991. Paper presented: *African trypanosomiasis: problems and research strategies.*

M. Juarrero

Cold Spring Harbour Meeting, Modern Approaches to New Vaccines, New York, USA, September, 1991. Poster presented: *Construction of recombinant capripox virus expressing the p67 kDa antigen of* Theileria parva.

R. Kaminsky

Food and Agricultural Organization (of the United Nations)/International Atomic Energy Agency Seminar for Africa on Animal Trypanosomiasis, February, 1991. Muguga, Kenya. Papers presented:

1. *Current status of* in vitro *assays for identifying drug-resistant trypanosomes.* R. Kaminsky.
2. *Preliminary studies on the uptake and efflux of radiolabelled drugs by susceptible and drug-resistant* Trypanosoma brucei brucei. R. Kaminsky, E. Zweygarth and R. Kratzer.

World Congress on Cell and Tissue Culture, June, 1991. Anaheim, USA. Paper presented: In vitro *studies on the mechanisms of multidrug resistance in African trypanosomes*. R. Kaminsky and E. Zweygarth.

17th International Congress of Chemotherapy, June, 1991. Berlin, Federal Republic of Germany. Paper presented: *Chemotherapy of African trypanosomiasis: studies on the mechanisms of multidrug resistance*. R. Kaminsky, E. Zweygarth and R. Kratzer.

S. Kemp

International Laboratory for Research on Animal Diseases Bovine gene mapping workshop, International Laboratory for Research on Animal Diseases, Nairobi, Kenya, February, 1991. Paper presented: *ILRAD gene-mapping group*. S.J. Kemp and A.J. Teale.

Food and Agricultural Organization (of the United Nations) Expert Consultation on the Genetic Aspects of Trypanotolerance. September, 1991. Food and Agricultural Organization (of the United Nations), Rome, Italy. Paper presented: *ILRAD Laboratory report*.

W. Kitungu

Federation of American Societies for Experimental Biology 75th Annual Meeting, Atlanta, USA, April, 1991. Paper presented: *The vacuolar ATPase of* Trypanosoma brucei. W.A.M. Kitungu and W. Fish.

R. Kruska

The Third Seminar on Land and Geographical Information Systems in Zimbabwe, March, 1991, Harare, Zimbabwe. Paper presented: *The development and use of geographic information systems to study tick-borne diseases of livestock in Zimbabwe*. R.L. Kruska and B. Perry.

S. Leak

Organization of African Unity/International Scientific Council for Trypanosomiasis Research and Control 21st Meeting, Yamoussoukro, Cote d'Ivoire, October, 1991. Paper presented: *Responses of N'Dama × Boran crossbred cattle to experimental infection with* Trypanosoma congolense. A.G.A. Leak, S.J. Kemp and A.J. Teale.

J. Lonsdale-Eccles

University of California, Los Angeles, Keystone Symposium, Keystone, Colorado, USA, May, 1991. Paper presented: *Characterization of the proteases of* Trypanosoma congolense *and* T. brucei. J.D. Lonsdale-Eccles, G.W.N. Mpimbaza, Z.R. Mbawa and M.J. Komblatt.

P. Majiwa

Molecular Parasitology Meeting, MBL, Woods Hole, MA, USA, September, 1991. Paper presented: *Gene amplification and rearrangement in* T. congolense. P.A.O. Majiwa, E. Omolo, E. Gobright and W.R. Fish.

M. Mamman

Tanzanian Veterinary Association Scientific Meeting, December, 1991. Arusha, Tanzania. Paper presented: *Sensitivity to diminazene and frequency of diminazene-resistant trypanosomes in populations of* Trypanosoma congolense *arising in animals following treatment with diminazene.* M. Mamman, N.B. Murphy, S. Kemei and A.S. Peregrine.

R. Masake

World Veterinary Congress, Rio de Janeiro, Brazil, August, 1991. Papers presented:

1. *Tsetse-transmitted African trypanosomiasis: its impact and control.* R.A. Masake, V.M. Nantulya and S.K. Moloo.
2. *Traditional role of women in animal care.* R.A. Masake.

International Scientific Council for Trypanosomiasis Research and Control, Yamoussoukro, Cote d'Ivoire, October, 1991. Papers presented:

1. *Characterization of* Trypanosoma congolense *stocks using molecular karyotyping.* R. Waihenya, R.A. Masake and G.K. Kinoti.
2. *Diagnosis of African trypanosomiasis.* R.A. Masake.

African Dissertation Internship Award Programme Workshop on Scientific Research in Africa, African Academy of Sciences, Naivasha, Kenya, September 1991. Paper presented: *Why should research be done in Africa?* R.A. Masake.

International Development for Research Cooperation/African Institute for Scientific Research and Development Workshop, Nanyuki, Kenya, June, 1991. Paper presented: *An overview of selected animal diseases.* R.A. Masake, P.N. Nyaga and T. Wachira.

D. McKeever

British Society for Immunology Spring Meeting, Workshop on Epitope Mapping, London, UK, April, 1991. Paper presented: *Mapping of T-cell epitopes on* Trypanosoma brucei *variable surface glycoprotein.*

Z. Mbawa

University of California, Los Angeles, Keystone Symposium. Keystone, Colorado, May, 1991. Paper presented: *Stage-specific expression of endopeptidolytic activity in African trypanosomes.* Z.R. Mbawa, I.D. Gumm, W.R. Fish and J.D. Lonsdale-Eccles.

S. Moloo

Organization of African Unity/International Scientific Council for Trypanosomiasis Research and Control 21st Meeting, Yamassoukro, Cote d'Ivoire, October, 1991. Poster presented: *Vector competence of different* Glossina *species for* Trypanosoma vivax, T. congolense *and* T. b. brucei. S. Moloo.

S. Morzaria

Gordon Research Congress, New Hampshire, USA, August, 1991. Paper presented: *A complete restriction map of the* Theileria parva *genome.* S.P. Morzaria and J.R. Young.

IV International Congress on Malaria and Babesiosis, Rio de Janeiro, Brazil, August, 1991. Paper presented: *New methods for the diagnosis of* Babesia bigemina *infection.* S. Morzaria, J. Katende, A. Kairo, V. Nene and A. Musoke.

Organization of African Unity/Food and Agricultural Organization (of the United Nations)/International Laboratory for Research on Animal Diseases Workshop on Tick and Tick-Borne Disease Control, Kampala, Uganda, September 1991. Paper presented: *Characterization and diagnosis of* Theileria.

A. Mukhebi

The Organization of African Unity/Food and Agricultural Organization (of the United Nations)/International Laboratory for Research on Animal Diseases Conference on Tick and Tick-Borne Disease Control in Eastern, Central and Southern Africa, Kampala, Uganda, September, 1991. Paper presented: *Economic modelling for tick-borne disease control.* A.W. Mukhebi and B.D. Perry.

A. Musoke

Food and Agricultural Organization (of the United Nations)/Danish International Development Agency Meeting on Immunization against *Theileria parva* and other tick-borne parasites, Kampala, Uganda, September, 1991. *Immunity to* Theileria parva *and other tick-borne parasites.*

D. Nandan

Molecular Parasitology Meeting, Molecular Biology Laboratory, Woods Hole, MA, USA, September, 1991. Paper presented: *Identification of molecule chaperones in African trypanosomes.* D. Nandan, E. Authié, Y. Verjee and T.W. Pearson.

V. Nene

The Jacques Monod Conference on Molecular Understanding and Control of Parasitic Diseases, Roscoff, France, July, 1991. Paper presented: *Protection of cattle against*

East Coast fever by a recombinant sporozoite antigen of Theileria parva. V. Nene, S. Morzaria, A.J. Musoke, E. Jones and C. Nkonge.

Gordon Research Conference on Molecular and Biological Aspects of Parasitism, New Hampshire, USA, August, 1991. Poster presented: *A recombinant* Theileria parva *sporozoite antigen protects cattle against East Coast fever.* V. Nene.

O. ole-MoiYoi

American Society of Microbiology Annual Meeting, Dallas, USA, May, 1991. Symposium on immunity to intracellular protozoal parasites. Paper presented: Theileria parva, *an intracellular protozoal parasite which transforms lymphocytes.*

Consultative Group on International Agricultural Research Centres' Day, International Centre for Research in Agroforestry, Nairobi, November, 1991. Paper presented: *Applications of biotechnology to agricultural development in Kenya.*

R. Pellé

21st Trypanosomiasis Seminar, Antwerp, Belgium, December, 1991. Paper presented: Trypanosoma brucei brucei *medRNA: stage-specific polyadenylation and association with cytoplasmic proteins.*

A. Peregrine

21st Meeting of the International Scientific Council for Trypanosomiasis Research and Control (International Scientific Council for Trypanosomiasis Research and Control), October, 1991. Yamoussoukro, Cote d'Ivoire. Papers presented:

1. *Towards understanding the molecular basis of drug resistance.* N.B. Murphy, G. Ndoutamia, R. Jamnadass and A.S. Peregrine.
2. *Occurrence of multidrug-resistant* T. congolense *populations in Ghibe, Ethiopia.* V. Codjia, W. Mulatu, E. Authié, S. Leak, J.R. Rowlands, G. d'Ieteren and A.S. Peregrine.
3. *Pharmacokinetics of diminazene in Boran* (Bos indicus) *cattle.* M. Mamman, Y.O. Aliu and A.S. Peregrine.

B. Perry

The Meetings of the International Society for Veterinary Epidmiology and Economics, Ottawa, Canada, August, 1991. Paper presented: *The determination of target populations for immunization against East Coast fever in two contrasting areas using integrated epidemiological and socioeconomic variables.* S.K. Maloo, S.K. Mining, B.D. Perry, J.J. Curry, J.M. Delehanty, R.L. Kruska, A.W. Mukhebi, W. Thorpe, S.M. Williamson and A.S. Young.

M. Sileghem

11th European Immunology Meeting, Helsinki, Finland, June, 1991. Paper presented: *Suppression of T-cell responsiveness during tsetse-transmitted trypanosomiasis in cattle.* M. Sileghem and J.N. Flynn.

V. Taiwo

42nd Meeting of the American College of Veterinary Pathologists, Orlando, Florida, USA, December, 1991. Paper presented: *Comparative* in vitro *macrophage erythrophagocytosis during the acute stages of bovine trypanosomiasis.* V.O. Taiwo, D.J.L. Williams, L.L. Logan-Henfrey and V.O. Anosa.

A. Teale

International Laboratory for Research on Animal Diseases Bovine gene mapping workshop, ILRAD, Nairobi, Kenya, February, 1991. Paper presented: *Mapping on trypanotolerance.* A. Teale.

D. Williams

International Scientific Council for Trypanosomiasis Research and Control 21st Meeting. Yamoussoukro, Cote d'Ivoire, October, 1991. Papers presented:

1. *Experimental infection with a haemmorrhage-causing* Trypanosoma vivax *in N'Dama and Boran cattle.* D.J.L. Williams, L. Logan-Henfrey, E. Authié, S.K. Moloo, J.M. Kabata, F. McOdimba and M. Opollo.
2. *Antibody responses to a parasite cysteine protease in cattle infected with* Trypanosoma congolense. E. Authié, Z. Mbawa, D. Muteti, P. Webster, J. Lonsdale-Eccles and D.J.L. Williams.

A. Young

Uganda Veterinary Conference, Kampala, Uganda, April 1991. Paper presented: *Chemotherapy of Theileriosis.* A. Young.

Organization of African Unity/Food and Agricultural Organization (of the United Nations)/International Laboratory for Research on Animal Diseases Workshop on Ticks and Tick-borne Diseases, Kampala, Uganda, September, 1991. Paper presented: *Vaccines against ticks.* A. Young.

SHORT-TERM VISITING SCIENTISTS

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Name	Project	Dates
Dr. S. Mahan College of Vet. Medicine Florida University Gainesville, USA	Phenotyping *Cowdria* cells	3/1/91–17/1/91
Dr. B. Allsopp Molteno Institute Cambridge, UK	*Theileria* gene probes	26/5/90–30/6/90 5/1/91–22/2/91
Dr. S. Kawazu International Institute of Animal Health Tsukuba, Japan	Molecular characterization of *Theileria* parasites	15/1/91–9/3/91
Dr. R. Huss-Ashmore Anthropology Dept. Pennsylvania University Philadelphia, USA	Social anthropology	18/1/91–1/7/91
Mr. P. Wijngaard University of Utrecht The Netherlands	Genes for *T. parva* antigens	25/1/91–24/2/91
Mr. A. Boulange IEMVT France	Trypanosome protein sequences	11/2/91–30/11/91
Prof. L.G. Tilney Department of Biology Pennsylvania University USA	Biology of *Theileria parva*/EM	15/2/91–15/4/91
Mr. R. Beecroft University of Victoria BC, Canada	Parasite protein sequences	22/2/91–22/3/91
Prof. V. Anosa Vaculty of Vet. Medicine University of Ibadan Nigeria	Haemopoiesis Ph.D. supervision	4/3/91–16/3/91
Dr. T. Obata TARC Tsukuba, Japan	Discussions with ILRAD in regard to cooperative research programmes	9/3/91–20/3/91

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Dr. W.K. Jorgensen Tick Fever Res. Centre Wacol, Queensland Australia	Diagnostics for protozoa	1/4/91–31/5/91
Mr. M. Eisler Faculty of Vet. Med. Glasgow University Scotland	Isometamidium ELISA	6/4/91–5/5/91 26/10/91–23/11/91
Mrs. E. Gault Faculty of Vet. Med. Glasgow University Scotland	Isometamidium ELISA	6/4/91–5/5/91 26/10/91–23/11/91
Dr. Susanna Williamson CTVM University of Edinburgh Scotland	Serological and chemical characterization of recent isolates of *Theileria*	8/4/91–7/8/91 5/9/91–31/10/91
Prof. P.H. Holmes Dept. of Vet. Physiology Glasgow University Scotland	Isometamidium ELISA/ Chemotherapy	29/4/91–6/5/91
Prof. G. Gettinby Dept. of Statistics and Modelling Sciences Strathclyde University Glasgow, Scotland	Consultant Statistician Disease and herd production models	11/5/91–25/5/91 16/9/91–30/9/91
Dr. R.L. Spooner IAPGR, Edinburgh Scotland	Consultations on collaborative work on bovine MHC class I reagents	30/5/91–1/6/91
Dr. P. Itty Institute of Agricultural Economics (ETH) Zurich, Switzerland	Socioeconomics of trypanosomiasis	1/6/91–30/6/91
Dr. J. W. Richardson Dept. of Agric. Econ. Texas A & M University College Station, Texas USA	Consultant: Simulation model economic impact of improved livestock disease control	4/6/91–16/6/91

Mr. P.T. Zimmel Dept. of Agric. Econ. Texas A & M University College Station, Texas USA	Modelling Expert: Simulation model economic impact of improved livestock disease control	4/6/91–16/6/91
Dr. H. Clevers Dept. of Clin. Immunol. Academic Hospital Utrecht The Netherlands	Use of COS cell transfection techniques	6/6/91–13/6/91
Dr. M. Desquesnes IEMVT Maisons-Alfort France	Serum analysis for protozoan infections in livestock in French Guyana	3/6/91–28/6/91 22/7/91–16/8/91
Ms. C. Seely Zoology Department Glasgow University Glasgow, Scotland	*T. vivax* antigens recognized by N'Dama and Boran sera	12/7/91–24/8/91
Prof. R. Terry Brunel University Uxbridge, Middlesex England	Training Adviser: Ph.D. supervision	1/9/91–15/9/91
Dr. J.A. Ellis University of Wyoming Laramie, Wyoming, USA	Cytokine assays in bovine trypanosomiasis	2/9/91–9/10/91
Dr. D.L. Berkvens Belgian Animal Disease Control Project Chipata, Zambia	Epidemiology modelling (ECFXpert)	15/9/91–22/9/91
Ms. A. Sherrif Strathclyde University Glasgow, Scotland	Tick database analysis	16/9/91–30/9/91
Dr. B. Goddeeris Louvain University Belgium	Cooperative research in immunology of theileriosis	18/9/91–24/9/91

Dr. James Ellis Natural Resources Ecology Laboratory Colorado State University Fort Collins, USA	Environmental studies	20/9/91–20/11/91
Dr. E. Galvin Natural Resources Ecology Laboratory Colorado State University Fort Collins, USA	Review of socioeconomic papers	20/9/91–20/11/91
Dr. R. Brun Swiss Tropical Institute Basel, Switzerland	Cooperative research on cultivation of trypanosomes	29/9/91–5/10/91
Dr. L. Wieler Institute of Hygiene & Diseases in Animals, Giessen, Germany	Monoclonal reagents for bovine leukocytes	28/10/91–12/11/91
Dr. D. Zilberstein Israel Institute of Technology Technion, Haifa, Israel	Cell biology studies of isometamidium uptake by trypanosomes	25/11/91–1/12/91
Dr. Coates Molecular Pathology Laboratory St. Thomas' Teaching Hospital London, England	RNA & DNA *in situ* hybridization	10/12/91–13/12/91
Ms. A. Waller Dept. of Immunology and Rheumatology St. Bartholomews Hospital Medical School London, England	Expression of cytokines and integrens	10/12/91–13/12/91