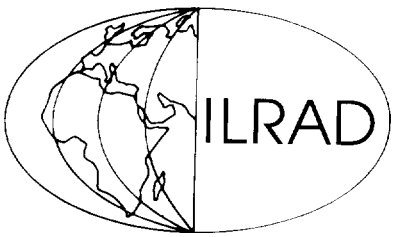
ILRAD 1992

Annual Scientific Report



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for Research on Animal Diseases

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PREFACE

The *Annual Scientific Report* was first published in 1987 to help bring the results of ILRAD's scientific programs more directly to the scientific community, especially those involved in the research and control of livestock diseases. This, the sixth such report, presents in the form of abstracts the results of the scientific work of the Laboratory in 1992. The project-based research management structure adopted by ILRAD in 1991 was fully implemented in 1992 and this report groups abstracts by research projects. The individual projects are listed below under the relevant program. The report also lists ILRAD's scientific papers for the year, the majority of which were published in refereed international scientific journals. In 1992, ILRAD also initiated an occasional, technical report series for publications of individual case-studies containing large data sets. These reports, like the ILRAD publications, are numbered and can be obtained on request from the ILRAD Library. A broader and less technical account of the operations and programs of the Laboratory can be found in the complementary publication, the *ILRAD Annual Report.*

TICK-BORNE DISEASES

In previous years, this program was principally confined to work on *Theileria parva*, the causative agent of East Coast fever in cattle, and was referred to as the Theileriosis Program. However, ILRAD has long been aware of the need to tackle the complex of tick-associated diseases which limit livestock productivity. Following approval by ILRAD's Board of Trustees for this expansion, the program now includes collaborative work on the diagnosis of anaplasmosis, babesiosis and other theilerial infections and the potentially protective antigens of the causative organisms, and is now entitled Tick-Borne Diseases. The major part of the work, however, still concentrates on epidemiology (E1), antigens (E2) and vaccine delivery systems for *Theileria parva* (E3). The eleven projects in this program cover:

- Characterization of *Theileria parva* (E1a)
- Development of new technologies for the detection of tick-borne diseases (E1b)
- Characterization of the genome of *Theileria parva* (E1c)
- Dynamics of the transmission of *Theileria parva* (E1d)
- Support for improved control of tick-borne diseases by national and regional programs (E1e)
- Antigens of tick-derived stages of *Theileria* and other tick-borne pathogens (E2a)
- Identification and characterization of antigens of *Theileria parva* schizonts that provoke cellular immune responses (E2b)
- Antigens of erythrocytic stages of *Theileria* and other tick-borne pathogens (E2c)
- Immunization with *Theileria* antigens (E3a)
- Evaluation of antigen delivery systems in cattle (E3b)
- Studies of cell-mediated immune responses of cattle (E3c)

TRYPANOSOMIASIS

The ten projects undertaken by the trypanosomiasis research staff were grouped in 1992 under two general headings, 'the epidemiology and biology of trypanosomes (T1)' and 'host resistance and immunity (T2)'. The projects were as follows:

- Epidemiology (T1a)
- Development and application of techniques for the diagnosis of African trypanosomiasis (T1b)
- Genetics of the livestock-infective African trypanosomes (T1c)
- Parasite differentiation: mechanisms involved in the control of cell division and proliferation of trypanosomes (T1d)
- Improved chemotherapy and chemoprophylaxis of trypanosomiasis in domestic livestock (T1e)
- Trypanosome antigens (T2a)
- The role of T cells and B cells in resistance to bovine trypanosomiasis (T2b)
- Significance of macrophage activation and function in the susceptibility of cattle to trypanosomiasis (T2c)
- Mechanisms of anaemia in bovine trypanosomiasis (T2d)
- The application of bovine genome analysis to the identification of markers and genes associated with trypanotolerance (T2e).

SOCIOECONOMICS

In 1992, the program addressed four major considerations formulated as individual projects:

- Production, delivery and adoption of alternative improved control measures for livestock diseases (ESE1)
- Economic, social and environmental impacts of livestock disease control (ESE2)
- Economics of alternative research approaches (ESE3)
- Relative economic importance of infectious diseases (ESE1)

Whilst work in projects ESE1 and 2 reflects a continuation of previous studies and is reported on here, projects ESE3 and 4 represent new initiatives which will be described in future reports.

We hope that this report will serve to illustrate the progress and scope of ILRAD's research programs for the wider research community and integrate further the work of the Laboratory with other laboratories and programs committed to the research and control of parasitic diseases of human and veterinary importance worldwide.

P.R. Gardiner
Information and Planning Officer
International Laboratory for Research on Animal Diseases
Nairobi, February 1993

THEILERIOSIS

E1a.1 Cloning of *Theileria parva*

Scientists: S. Morzaria, R. Bishop, A. Young, T. Dolan. E. Taracha
Research Associates: P. Spooner, F. Mwakima

Using a cloning method which has been described in previous ILRAD reports, single clones have been obtained from five *Theileria parva* stocks (cattle-derived Muguga, Marikebuni, Boleni and Uganda and buffalo-derived 7014) as sporozoite stabilates. Characterization using monoclonal antibodies (MAbs) and DNA probes *in vitro* shows clones from cattle-derived parasites to be similar to the bulk populations from which they were derived. The 7014 clone shows different restriction fragment length polymorphisms (RFLPs) from the bulk population when its DNA is hybridized with a TPR1 repetitive DNA probe.

Schizont-infected lymphocyte lines containing cloned parasites which show different RFLPs from the existing sporozoite clones when their DNA is hybridized with TPR1 probes have been identified from three *T. parva* stocks. In the case of the clones derived from the Marikebuni stock, different sizes of the polymorphic immunodominant antigen (PIM) have been identified by Western blotting. Attempts are under way to obtain additional sporozoite clones from within these stocks.

Immunological characterization of the cloned Marikebuni parasite has begun. Fourteen cattle were immunized with the cloned Marikebuni stabilate 3292. Seven were challenged with the Marikebuni bulk stabilate 3014 and the other seven with the Muguga bulk stabilate 3087. One out of the seven animals reacted severely in each of the challenge groups, suggesting that the cloned Marikebuni parasite may give less broad cross-protection than the parent bulk stabilate. The strain specificity of cytotoxic T lymphocytes (CTL), present in peripheral blood mononuclear cells of 8 of the 14 immunized animals, was also analysed. Six of the animals developed parasite-reactive CTL detectable in either bulk or limiting dilution culture assays. CTL from all six animals were capable of killing autologous cell lines infected with cloned or bulk Marikebuni parasites, but only two animals exhibited CTL capable of killing autologous cell lines infected with the bulk Muguga stock. This contrasts with previous experiments in which immunization of animals with the bulk Marikebuni stock always resulted in induction of CTL which killed both Marikebuni and Muguga targets. The finding that four of the eight animals were solidly immune to challenge, in the absence of detectable CTL specific for the parasite used for challenge, suggests that non-CTL mechanisms of protection may have been operating in these animals.

E1a.2 Studies of the sexual cycle of *Theileria parva*

Scientists: S. Morzaria, R. Bishop, J. Young, A. Young, T. Dolan
Research Associates: P. Spooner, F. Mwakima

A recombinant *Theileria parva* parasite derived from elements of the Muguga and Uganda stocks has been isolated from an animal experimentally co-infected with these two stocks. Recombinant sporozoite populations from ticks with a single infected acinus were identified by hybridization with oligonucleotides, derived from

TPR1 repetitive DNA sequences, which were specific for each of the two stocks. Populations hybridizing with both probes were considered to contain recombinant parasites. A cell line containing a cloned recombinant parasite was isolated after infection of bovine peripheral blood mononuclear cells at a ratio of less than one sporozoite per 100 lymphocytes and subsequent limiting dilution cloning of the infected cells. Analysis of the cloned cell line with anti-schizont monoclonal antibodies (MAbs) and DNA probes confirmed the recombinant nature of the parasite and were consistent with the recombinant having acquired two chromosomes from each parent. A sporozoite stabilate of the cloned recombinant parasite has been prepared after isolation of the parasite from ticks which were allowed to feed on an animal infected with the recombinant. The ability of animals immunized with the cloned stabilate to withstand challenge with the Uganda and Muguga stocks of *T. parva* is currently under investigation.

A putatively recombinant parasite derived from the Muguga and Marikebuni stocks has been isolated using a method similar to that described above. A cloned cell line infected with the recombinant parasite has a Muguga genotype when hybridized with repetitive and ribosomal DNA probes, and a Marikebuni type MAb profile. Hybridization with a telomeric DNA probe suggests that three chromosomes are derived from the Marikebuni parent. A sporozoite stabilate of the recombinant parasite is being prepared.

E1a.3 Polymorphic DNA markers for discrimination of *Theileria parva* parasites

Scientists: S. Morzaria, R. Bishop, V. Nene
Research Associates: P. Spooner, E. Gobright

Cloned *Theileria parva* repetitive, ribosomal and telomeric DNA sequences have been isolated and are useful for discrimination of *T. parva* stocks using restriction fragment length polymorphisms (RFLPs). These have been described in greater detail in a previous ILRAD report. Synthetic oligonucleotides derived from TPR1 repetitive DNA sequences have been developed for three stocks. These allowed +/– discrimination of the stocks without restriction and Southern blotting of DNA and made possible the laboratory isolation of recombinant parasites derived from these stocks.

The technique of polymerase chain reaction (PCR) amplification using single arbitrary primers (10-mers) has been tested on purified piroplasm DNA from different *T. parva* stocks. Certain arbitrary primers generate amplified fragments which are consistently polymorphic between different stocks. Due to the requirement for purified parasite DNA, the technique will not be useful for epidemiological studies. However, it may be possible to associate polymorphisms defined by arbitrary primers with phenotypes of interest, such as the strain specificity of the immunity induced by *T. parva* populations in cattle. This might provide an approach to the isolation of the genes encoding parasite antigens which are the target of the protective immune response in cattle.

Additional allelic markers are required for the study of recombination in field populations of *T. parva*. These will be provided by studies of polymorphism within

cloned antigen genes. Sequencing of the gene encoding the p67 sporozoite antigen from different *T. parva* stocks has revealed a 129 bp insertion in three buffalo-derived stocks which is absent from five cattle-derived stocks. It is possible that this additional sequence may provide a marker for *T. parva* from buffalo. To examine this possibility a study in which the whole p67 gene is amplified by PCR from both cattle-derived and buffalo-derived sporozoite populations has been initiated [see Abstract E1a.4].

E1a.4 Characterization of cattle- and buffalo-derived *Theileria parva* sporozoite stabilates using p67 primers as markers

Scientists: S. Morzaria, V. Nene
Research Associates: P. Spooner, E. Gobright

Sequencing of the p67 gene from different *Theileria parva* stocks has revealed a 129 bp insertion in two buffalo-derived stocks which is not present in the Muguga stock. Thus it is possible that this additional DNA sequence in the p67 gene may provide a marker for buffalo-derived *T. parva*. In order to examine this, DNA primers flanking the 129 bp sequence in the conserved region of the gene have been designed to characterize cattle- and buffalo-derived *T. parva* sporozoite populations [see Abstract E2a.1].

Conditions for isolating DNA from cryopreserved ground-up tick supernates containing sporozoites have been optimized and the DNAs used in PCR reactions using the primers described above. The results show that three sporozoite populations derived from naturally infected buffalo have a mixed p67 genotype of both cattle- and buffalo-derived *T. parva* sporozoites. However, cattle-derived parasites show a single PCR product corresponding to the size expected from a p67 gene without the 129 bp sequence.

A buffalo-derived sporozoite population which has been passaged through cattle and transformed will be examined. This investigation may answer the question of whether 'transformation' of *T. p. lawrencei* into *T. p. parva* is due to selection or genetic mutation.

E1b.1 Development of antigen and antibody capture ELISAs for babesiosis

Scientists: A. Musoke, S. Morzaria, A. Young, T. McGuire*, T. Dolan
Research Associates: J. Katende, R. Skilton
Research Fellow: T. Ntando
Technicians: A. Maina, L. Wangui, M. Omenya, A. Sore, T. Ndolo, S. Mwaura

*Washington State University, Pullman, Washington, USA

In an attempt to develop enzyme-linked immunosorbent assays (ELISA) for the detection of *Babesia bigemina*, sera collected from cattle resident in an endemic area were screened by Western immunoblotting to identify conserved antigens which would serve as the basis for development of the assay. Most of the sera recognized a major protein with a relative molecular weight of 200 kDa, while a few sera showed reactivity with minor proteins of 55 and 33 kDa. The 200-kDa protein was also recognized by sera from cattle experimentally infected with *B. bigemina*. This protein is not recognized by sera collected from cattle experimentally infected with *B. bovis*.

Monoclonal antibodies (MAbs) generated against the 200-kDa protein stained the surface of live parasites in the indirect immunofluorescent antibody test, but showed no reactivity with blood elements or the common haemoparasites of cattle, *B. bovis*, *Anaplasma marginale*, *Theileria parva*, *T. mutans*, *T. buffeli*, *Trypanosoma vivax*, *Trypanosoma brucei* and *Trypanosoma congolense*. Two of the MAbs, BbF4/86.9 and BbF4/86.34 were selected on the basis of their reactivity with epitopes not blocked by bovine immune sera and their recognition of independent epitopes on the molecule, for the development of antibody and antigen capture ELISAs. The assays detected parasite antigen in the sera of cattle experimentally infected with *B. bigemina* on day 6 following infection, while antibodies to the molecule were detected between day 11 and 13 and persisted to day 250. When the assays were used to analyse sera from cattle in endemic areas (Kenya, Ethiopia and French Guyana), the results indicated a prevalence of antigenaemia of between 40 and 60% while the antibody had a prevalence of greater than 70%. The two assays will prove useful in determining the true prevalence of *B. bigemina* in endemic areas and also to differentiate infections of *B. bigemina* from *B. bovis* in cattle.

E1b.2 Development of antigen and antibody capture ELISAs for anaplasmosis

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Research Associates: J. Katende, R. Skilton
Student: T. Ntando
Technicians: A. Maina, L. Wangui, M. Omenya, A. Sore, T. Ndolo, S. Mwaura

*Washington State University, Pullman, Washington, USA

A similar approach to that described for *B. bigemina* using field sera from cattle resident in endemic areas [see Abstract E1b.1] has been used to identify candidate antigens of *Anaplasma marginale*. Two prominent antigens, one of 23 kDa and one

of 75 kDa, were identified. Monoclonal antibodies (MAbs) generated against the two antigens stained the surface of live *A. marginale* initial bodies by indirect immunofluorescence. They did not react with blood elements or the common haemoparasites of cattle [see Abstract E1b.1]. MAbs Ana4/39.20 and Ana4/39.28 that recognized epitopes of the 23-kDa antigen were selected for the development of the antigen capture ELISA on the basis of recognizing epitopes not blocked by bovine immune sera and also in their ability to recognize independent epitopes on the molecule. The assay was used to analyse sera from endemic areas (Kenya, Uganda, Burkina Faso, Ethiopia and French Guyana), and the results obtained indicated an antibody prevalence against the 23-kDa molecule of between 95 and 100%. An experiment to ascertain the kinetics of antigenaemia in experimentally infected cattle has been initiated. This assay was compared with the ability to detect parasites using a DNA probe for *A. marginale* (from Washington State University, USA). Out of 160 serum samples collected from animals at Kapiti Plains Ranch, Kenya, 130 were positive for antigenaemia. Using the DNA probe, only 63 animals were found to contain *A. marginale* DNA. In order to ascertain that positive animals are indeed infected, one parasitologically negative but antigen-positive calf was splenectomized. *Anaplasma marginale* initial bodies were seen in Giemsa-stained blood smears from the animal ten days later. Another 11 calves which had been found negative by the antigen detection ELISA were also splenectomized and Giemsa-stained smears examined for 30 days. No *A. marginale* initial bodies were detected. Monoclonal antibody An4/36.13 is being used similarly to develop and validate the 75-kDa antigen for use in an antibody detection ELISA for *A. marginale*.

E1b.3 Nucleic acid technologies for the detection of *Theileria* species

Scientists: R. Bishop, S. Morzaria, A. Young
Research Associates: P. Spooner, E. Gobright

Three different types of probes are being evaluated. These are based on *Theileria* species-specific repetitive DNA sequences, small subunit ribosomal RNA sequences (SSUrRNA) and large subunit ribosomal RNA sequences (LSUrRNA).

Repetitive DNA sequences have been isolated from *T. parva, T. mutans* and *T. taurotragi*. The *T. parva* sequence has been used for detection of *T. parva* in tick salivary glands and primers derived from the repetitive sequence have been used to detect the parasite in the blood of carrier cattle using polymerase chain reaction (PCR) amplification. The *T. mutans* repetitive sequence can detect parasitaemias as low as 0.4% by hybridization to whole blood DNA from infected cattle. Using primers derived from the repetitive sequence allowed detection of parasitaemias in cattle of < 0.1%. Repetitive sequences isolated from a *T. taurotragi* genomic library do not hybridize to DNA from all available *T. taurotragi* isolates. A major problem with the repetitive probes is their lack of conservation within a species.

Sequencing of the DNA of SSUrRNA 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 either by probing ribosomal DNA amplified from whole genomic DNA using the PCR, or parasite ribosomal RNA in schizont-infected lymphocytes. Preliminary indications are that the oligonucleotides can also be used for detection of *Theileria* rRNA in tick salivary glands, but this technique requires optimization.

DNA sequencing also reveals species-specific regions within LSUrRNA genes, which are larger than those within SSUrRNA genes, although not all are conserved within a species. Oligonucleotides which differentiate *T. taurotragi* and *T. parva* have been developed and development of specific oligonucleotides for other *Theileria* species is in progress. These will be used in combination with oligonucleotides derived from SSU sequences to improve sensitivity.

Preliminary work has been carried out to adapt the probes for non-radioactive use. Labelling of repetitive probes with dioxigenin was effective but was less sensitive than radioactivity when a colour reaction was used for detection. Biotinylated ribosomal oligonucleotides were used successfully to detect PCR amplified ribosomal DNA.

E1c.1 Comparative restriction maps of *Theileria parva* stocks

Scientist: S. Morzaria
Technician: T. Ndolo

Studies of the comparative *Sfi*I physical maps of the three immunologically important stocks of *Theileria parva*, namely Mariakani, Marikebuni and Uganda, require the preparation of low melting point agarose-embedded high molecular weight genomic DNA obtained from infected cattle having a piroplasm parasitaemia of greater than 15%. These DNAs have now been prepared from groups of cattle infected with the relevant working stock stabilate. The DNAs have been analysed in preliminary studies for their suitability for *Sfi*I restriction digests. Southern blots of the *Sfi*I digested DNAs from these stocks are now currently being prepared. In total, 90 Southern blots of pulsed-fied gel electrophoresis (PFGE) separated *Sfi*I fragments will be required for probing with all the available linking clones and other gene markers.

Methods for constructing *Sfi*I linking maps of parasite stocks that do not produce high piroplasm parasitaemia have been evaluated. The most suitable approach utilizes the preparation of high molecular weight agarose-embedded DNA from schizont-infected cell lines. The ethidium bromide stained gels of such *Sfi*I digested DNA in PFGE show a DNA smear because of gross contamination with bovine genomic DNA. However, Southern blots of such gels, when probed with linking clones, show distinct bands which can be identified using *Sfi*I-digested DNA from control piroplasms. This technique will be utilized for determining the comparative physical maps of *T. parva* Boleni and *T. parva* 7014 buffalo-derived stocks.

Construction of a *T. parva* P1 library representing the whole genome has begun using two P1 cloning vectors—AD10 and a new derivative *SacBI*I. Both vectors have been successfully used by other workers for the construction of recombinant libraries of ~100 kb fragments from human as well as *Drosophila* genomic DNA. The AD10 vector contains a unique *BamH*I and *Sal*I sites located within a tetracycline-resistance gene (originally from pBR322). Thus cloning of inserts into the AD10 vector inactivates the tet-R gene and makes the colony sensitive to tetracycline. The *SacBI*I vector, a derivative of AD10, is a positive vector allowing only those P1 clones containing inserts to survive when the cells are plated on sucrose. Several approaches have been attempted to produce high molecular weight genomic DNA (between 75 and 170 kb) from piroplasms of cloned *T. parva* Muguga stock. The successful approach utilized size-selected *Sau*3A partially-digested DNA separated on PFGE. This DNA is currently being evaluated for suitability for cloning in both the P1 vectors described above.

E1c.2 Telomeric sequences of *Theileria parva*

Scientists: R. Bishop, S. Morzaria
Research Associate: P. Spooner

An oligonucleotide specific for telomeric regions of *Plasmodium berghei* has been used to isolate a 1.8 kb *Theileria parva* telomeric DNA sequence from a sheared DNA

library. The clone contains approximately 600 bp of simple telomeric repeats and 1200 bp of subtelomeric sequence which does not contain significant repeated sequences. The probe specifically recognizes *Theileria* telomeric sequences and not bovine telomeres and when hybridized to DNA from schizont-infected lymphocyte cultures is useful for the analysis of cloned and recombinant *T. parva* parasites and also field isolates. By screening with a probe containing only subtelomeric sequences from the original clone, overlapping bacteriophage clones containing approximately 14 kb of subtelomeric sequences derived from the largest *T. parva* chromosome have been isolated. Preliminary characterization of the cloned subtelomeric DNA suggests the absence of repetitive DNA sequences in large quantity, although there is some homology between a subset of telomeres up to approximately 5 kb, 5′ to the simple telomeric repeat sequences.

In future it will be necessary to complete the characterization of the existing subtelomeric clones and test for the presence of transcribed sequences on Northern blots. Sequences upstream of the existing subtelomeric clones will be characterized using purified telomeric *Sfi*I fragments and P1 clones.

E1c.3 MDR gene family of *Theileria parva*

Scientists: O. ole-MoiYoi, A. Peregrine, P. Neubald*
Student: M. Kibe

*Brunel University, UK

A DNA sequence encoding a P-glycoprotein from *P. falciparum* was used to screen a library of *T. parva* genomic DNA constructed in λgt11. Among the 30 positive clones identified, more than 75% hybridized to each other. The majority of these clones contained a 750 bp DNA fragment. The sequence of one of these clones revealed regions coding for nucleotide-binding domains with homology to those of higher eukaryotic MDR genes. This fragment of DNA was used as a probe to isolate clones with larger inserts. A clone containing a 3.35 kb DNA fragment, which covers the entire *T. parva* P-glycoprotein gene, has been completely sequenced. This gene sequence has seven introns, the smallest of which is 29 bp while the largest is 84 bp long. A cDNA of about 3 kb has also been identified by screening a cDNA library constructed from infected lymphocyte mRNA. This cDNA has a 600 bp, 5′ domain which is unusual in that its AT content is 37%, unlike any *T. parva* genes reported to date. The origin of this 5′ GC-rich region is not yet known, since it does not hybridize to either *T. parva* or lymphocyte DNA. This clone also has retained the most 5′ of the introns. Interestingly, the clone has a poly A tail. An additional cDNA was sought by re-screening the cDNA library. A clone with an insert of about 2.3 kb was isolated and has been partially sequenced. A sequence of the 3 kb cDNA shows, over the region of identity between the two clones, slight differences from the 2.3 kb cDNA. An open reading frame from the latter clone, which is about 1.7 kb, has been expressed in the expression vector pUR291. Fusion proteins have been prepared and purified on preparative polyacrylamide gels for immunization of rabbits.

E1d.1 Development of *Theileria parva* in the tick

Scientists: A. Young, S. Morzaria, T. Dolan
Research Associate: F. Mwakima
Technicians: S. Mwaura, G. Njihia, M. Muthoni

The control of infection with *Theileria parva* in the tick *Rhipicephalus appendiculatus* is multifactorial. The duration and degree of parasitaemia, the number of differentiated gametes, the sex and instar of the tick and the environmental temperature have all been implicated in influencing the infection rate in the tick. Currently, the nature of infection in cattle as a variable parameter affecting infection rates in ticks is being investigated.

There is evidence that one of the major factors controlling the infection rate in ticks is the piroplasm parasitaemia at the time the nymph ticks feed. Many doses of sporozoite stabilate cause either peracute or mild infection resulting in short periods of piroplasm parasitaemia. In order to optimize conditions for tick isolation of parasites, studies have been performed using one stabilate of *T. parva* Muguga (Stabilate 3087), one stock of *R. appendiculatus* and age-matched Boran cattle from a single source. Because of previous variability in the source of infection for ticks, this may confound analysis of the tick database reported elsewhere. Drug treatment has been used to manipulate the course of infection. A comparison of treatment with a short-acting oxytetracyline formulation from days 10–14 after infection and dexamethasone on days 13 and 15 after infection, either in combination or separately, has been used. Dexamethasone on its own reduces pyrexia and increases piroplasm parasitaemia and may prolong infection. Oxytetracycline on its own prolongs the duration of infection, can increase recovery rate and reduces pyrexia. When used in combination the treatment increases the piroplasm parasitaemia, reduces pyrexia and prolongs the infection.

On analysis of tick infection in these experiments, there was no evidence that infection levels increased after dexamethasone treatment although higher parasitaemias developed. However, with the use of oxytetracycline treatment, it was possible to increase the dose of *T. parva* (Muguga) stabilate from 1/100 to 1/20 dilution, thereby producing more uniform infections in cattle. Treated animals have a more prolonged infection allowing the production of a greater number of tick batches per animal. Analysis of infections in ticks produced from 28 cattle over a six-month period has shown a linear relationship between parasitaemia and infection in ticks. However, this only applied to the early part of infection (up to day 17 after infection). The highest infections occur in ticks which complete engorgement as nymphs on day 17, although ticks which drop on days 18 and 19 can also be highly infected. Fifty-eight percent of tick batches which become replete on days 17, 18 or 19 reached a *T. parva* abundance of 80 infected acini or more per tick—which is the required level for efficient harvesting of *T. parva* sporozoites. This compares with 35% of tick batches which reached this level of infection between day 16 and 22 after infection and 25% between 15 and 24 days.

This is a major improvement since only 5% of the tick batches reached this infection level previously. It should now be possible to design a feeding system where large numbers of engorged ticks are produced during the period of infection when

cattle are most infective to ticks. This should in turn reduce the work required to provide a continual supply of highly infected ticks for research and reduce the requirements for cattle. The implication of these studies is that the infectivity of piroplasms to the tick is time dependent which is likely to reflect the concentration of gametocytes infective to ticks and, possibly, the cattle reaction to the piroplasms. Rising piroplasm parasitaemias, with between 2 and 20% of erythrocytes infected, appear to be the most infective to ticks, while peak or falling parasitaemias appear less infective.

E1d.2 Optimization of feeding techniques for the production of infected ticks *in vitro*

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Research Associate: F. Mwakima
Technicians: S. Mwaura, G. Njihia, S. Ochieng'*

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Two approaches to the feeding of ixodid ticks *in vitro* have been taken. The successful feeding of ixodid ticks to repletion *in vitro* was not reported until 1991 when ICIPE scientists were successful in feeding adult *R. appendiculatus* on an artificial Baudrache membrane with tactile and olfactory stimuli added. In collaboration with ICIPE, the technique has now been applied to *R. appendiculatus* nymphs. The nature of the anticoagulant method for the blood meal for the tick was investigated. Heparinized blood was found to be better for tick feeding than the original defibrinated blood, and blood with acid citrate dextrose and EDTA was not suitable. The size of membrane fed nymphal ticks was comparable to those fed on cattle, as was their feeding and moulting periods. It was demonstrated that heparinized and defibrinated blood taken from *T. parva*-infected cattle was infective to ticks fed on membranes and the resultant adult ticks could transmit *T. parva* to cattle. The infective blood only needs to be provided from the day before the ticks become replete and detaches. Defibrinated blood was not as infective to ticks as heparinized blood. When the infection levels in adult ticks fed on membranes as nymphs was compared with those of ticks fed on blood donor cattle infected with *T. parva*, it was found that infection levels were, on occasion, comparable but were usually lower in the membrane fed ticks. This was probably due to the nature of the presentation of the blood to the feeding tick.

Using the present system, up to 4,000 nymphal ticks can be fed on membranes in one experiment. An improved apparatus for feeding ticks has been developed which automatically circulates, cools and heats the blood and, with further development, could be useful for maintenance and infection of ticks with pathogens.

In another approach, skin membranes prepared from cattle or rabbits have been used to feed all instars of *Amblyomma variegatum*. The stimulus important for the feeding of this species is a 5 to 10% concentration of carbon dioxide in the atmosphere. This is not required for *R. appendiculatus*. Ticks fed this way have

transmitted *T. mutans* and *Cowdria ruminantium*. It is likely that with the provision of the required stimuli, it will be possible to feed most species of ixodid ticks on membranes.

E1d.3 Infection levels in male, female and nymphal ticks and survival of parasites in ticks

Scientists: A. Young, S. Morzaria, T. Dolan, M. Shaw
Research Associate: F. Mwakima
Research Fellow: H. Ochanda
Technicians: S. Mwaura, G. Njihia

Male ticks normally have much lower infections of *T. parva* in their salivary glands than female ticks. Although they can often reach the same percentage of infected ticks as females, they rarely reach the abundance or intensity of infection obtained in the female. Analysis of the infection levels in female ticks show that on average they have three times higher infection than male ticks. The same phenomenon is seen in *R. appendiculatus* infected with *T. taurotragi*, although in *T. mutans*-infected *A. variegatum*, males are more highly infected. Ultrastructural and morphological studies are being undertaken to determine why this is the case. A simple explanation was obtained from the study of the salivary glands of males and female *R. appendiculatus* ticks. On average female ticks had 1,736 type III salivary gland acini compared to 1,346 acini in the male tick. Type III acini are the only acini reported to be infected with *T. parva*. Because the difference in infection level in male and female ticks is greater than the difference in salivary gland acini number, other differences in the ticks should be considered.

When the infections developing in nymphs were compared to adult ticks which had become replete on *T. parva*-infected cattle on the same day, it was found that nymphs often produced the same infection rate as adult ticks. However the abundance of the infection in adults was approximately 20 times higher than in the nymphs. This directly reflected the greater number of type III acini in the adults compared to the nymphs (87 type III acini). In the field, however, this is compensated for by the greater number of nymphs feeding on a host animal.

Using the Muguga stock of *R. appendiculatus* and *T. parva*, the effects of different temperatures on the development of the sexual stages within the gut of nymphal ticks have been investigated. Engorged nymphal ticks maintained at 28 °C, 20 °C and under natural conditions at ILRAD proved no more favourable in producing higher infections than a standard temperature of 24 °C and often were less favourable.

The effects of different temperatures on the decay of *T. parva* Muguga infection in the salivary glands of adult ticks was investigated. It was found that salivary gland infections die off rapidly at 28 °C and more slowly at 24 °C and 20 °C, but under natural conditions higher infections developed and decayed much slowly than under constant temperature in the laboratory. In a second experiment, ticks of high and low infection are being exposed to a variety of conditions under natural and laboratory conditions.

E1d.4 Determination of the effects of tick diapause on the transmission of *Theileria parva*

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Behavioural diapause in adult ticks is apparently widely distributed in Southern African ticks. This diapause is expressed by inactivity of the adult ticks for several months of the year so that ticks do not attach to hosts. It is believed to be a strategy for survival during the long, hot and dry period prevalent in these areas and results in seasonal infestation of hosts by tick instars and a seasonal incidence of theileriosis. It is likely that diapause is controlled by changing day length (photoperiod) in these areas. Diapause is an important feature of tick population dynamics and transmission of theileriosis. Because little work has been carried out on diapause in *R. appendiculatus*, a series of experiments was undertaken to investigate the presence and effect of diapause.

In two experiments at ILRAD, diapause was studied in the laboratory by exposing adult ticks from East, Central and South Africa to 10 hours light/14 hours dark, 14 hours light/10 hours dark or continuous dark. It was found that none of these conditions had any effect on *R. appendiculatus* populations from East Africa as judged by attachment and feeding on rabbits. In contrast, Central and Southern African colonies of *R. appendiculatus* fed much more efficiently when exposed to 14 hours light/10 hours dark. Hence it appears that Central and Southern Africa populations have the ability to undergo behavioural diapause but an East African population does not. Studies on the closely related *R. zambeziensis* from Zimbabwe gave an inconclusive result.

An experiment involving exposure of ticks in the field is being undertaken in collaboration with workers in Zimbabwe and Kenya. Engorged female ticks were collected from Kiambu District, Kenya, Western Mashonaland, Zimbabwe, and East Province, Zambia. The three stocks of *R. appendiculatus* have been exposed to natural conditions at ILRAD and at the Veterinary Research Laboratories at Harare as larvae and nymphae and then fed on rabbits. The adults, in nylon mesh tubes, have now been exposed at both sites and the behaviour of the ticks is being observed. In addition the ticks are being applied to rabbits at monthly intervals to determine whether ticks are in diapause or not.

E1d.5 Interactions between sporozoites and host cells

Scientists: M. Shaw, A. Musoke, L. Tilney*, A. Young

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The role of Ca^{2+} in the entry of sporozoites into lymphocytes has been examined. It was found that depletion of Ca^{2+} from the external medium by using EDTA, EGTA or BAPTA (calcium chelating agents) had no inhibitory effect on sporozoite entry. However, a number of Ca^{2+} channel blockers and calmodulin antagonists induced a reversible, dose-dependent, inhibitory effect. In addition, sporozoite entry could be inhibited by treatments expected to inhibit the intracellular release of Ca^{2+}, suggesting that the mobilization of intrasporozoite Ca^{2+} is essential for parasite entry. Attempts to localize Ca^{2+} within the sporozoites using cytochemical techniques have not been successful presumably because the level of Ca^{2+} present is below the level of detection of the methods used.

In other experiments, the possibility that the invasion of lymphocytes by sporozoites may involve interactions with sulphated glycoproteins of the host cells surface is being explored. Initial results show that sporozoite entry can be significantly inhibited in a dose-dependent manner by arginine-glycine-aspartic acid tripeptides, fibronectin and by antibodies reactive with fibronectin. Furthermore, sporozoite entry was inhibited by heparin but not by hyaluronic acid or laminin. Hyaluronic acid is a highly carboxylated, high molecular weight carbohydrate and suggests that the observed inhibition of sporozoite invasion was not primarily the result of non-specific charge effects.

The process of establishment of newly entered sporozoites within the lymphocytes has been examined in detail as a prelude to determining fate of the surface molecules of the sporozoite during the entry process. The newly entered sporozoite rapidly escapes from the enclosing host cell membrane and this process is accompanied by the discharge of the rhoptries and microspheres as well as the rapid elaboration of membrane systems derived from the sporozoite nuclear envelope. The discharge of the secretory organelles is not a pH-induced process. Immunoelectron microscopic localization studies are now in progress to examine the fate of the sporozoite surface molecules p67 and p85 during the process of entry and establishment of infection.

E1d.6 Studies of the transmission of *Theileria parva* by ticks to cattle

Scientists: M. Shaw, A. Young
Student: E. Kimbita
Research Associate: F. Mwakima

It has been shown that male ticks produce fewer sporozoites per 'e' cell in type III acini than females in light microscopic studies. In ultrastructural studies, the development of sporozoites in the male is seen to take longer than in the female and the 'e' cell does not become so enlarged. A comparison is now being made between the development of sporozoites in nymphal and adult ticks.

Electron microscopic studies have demonstrated that sporozoites are released from infected 'e' cells as a trickle rather than in a burst. This is being complemented by light microscopic studies to see if there is a sequence of release of sporozoites into the salivary ducts within the salivary gland.

The development of sporozoites within the tick 'e' cell in male and female adult ticks in response to different stimuli, particularly high temperature, and at different times after moulting is being examined using the electron microscope and is being correlated with infection *in vitro*. Differences in the numbers of sporozoites and the degree of maturation between male and female ticks fed on rabbits have been noted which correlated with infectivity *in vitro*. In ticks aged 42 days post-moult or older, high temperature (37 °C) for up to 10 days was not sufficient on its own to induce complete sporozoite maturation and no infections were established *in vitro*. Comparison of the effects of high temperature on sporozoite maturation in adult ticks at earlier times post-moult are in progress.

E1d.7 Tick database analyses and modelling of theileriosis

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The Tick Unit database was established in 1986. By July 1991, records of 286 animal infections and 1,241 tick batches harvested from infected animals had been stored. The factors influencing clinical and parasitological parameters in Boran cattle after experimental infections with stabilates of *Theileria parva* Muguga and factors influencing infection in ticks fed on these cattle were investigated by analysis of these records. The results of the general linear model analyses indicate that stabilate/dilution has an influence on the time required before detection of schizonts, time to febrile response, minimum packed cell volume, minimum white blood cell count, and duration of febrile response of surviving animals (but not on time to detection of piroplasms, duration of patent schizonts of surviving animals, duration of piroplasms of surviving animals and time to recovery). The sex of the animal has an influence on time to detection of piroplasms, minimum packed cell volume and time to recovery. The age of animal influenced minimum packed cell volume as did pen temperature. Administration of dexamethasone influenced the duration of patent schizont parasitaemia in surviving animals and time to recovery. The duration of piroplasm parasitaemia was the only variable found not to be influenced by any factor. Most factors have a significant effect on prevalence of infection in ticks and the intensity, abundance and variability of abundance of such infections. The most influential factors in approximate order of importance were piroplasm parasitaemia of the animal on the day of harvest, minimum packed cell volume, age of animal, sex of animal, stabilate/dilution and whether the animal recovered from infection. Temperature of the pen had some effect on intensity and abundance in male and female ticks. Tick stock affected prevalence in males and females, and abundance in male ticks.

The risk factors that contribute to the severity of infection in animals infected with the *Theileria parva* Muguga stabilate were also explored. When the risk factors considered were based on measurements taken at the time of inoculation, logistic regression analyses revealed that only one factor influenced the probability of recovery and the probability of a severe reaction. This factor was the stabilate/dilution combination administered to the animal. The findings are based on statistical significance. The next step will be to refine these analyses and examine them for biological significance.

E1d.8 Preliminary analysis of the transmission dynamics of *Theileria parva* in eastern Africa

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Two mathematical models were developed that investigate the transmission dynamics of *Theileria parva* by *Rhipicephalus appendiculatus* to cattle in endemically stable areas. A method of estimating the rate of infection of cattle with *T. parva* at the endemically stable state is given. Empirical estimates of all the parameters in the model are available. The degree to which animals that have recovered from theileriosis (the 'carrier' state) are able to transmit infection to nymphs as larvae is a crucial determinant of the dynamics of infection in a herd.

Two methods influencing the transmission of infection are considered, infection-and-treatment immunization and reduction in the success of tick-feeding rate by acaricide. The impact of each method on the transmission of infection was evaluated and criteria for control by vaccination were derived. The data that will be required which is necessary to predict the dynamics of *T. parva* infection in cattle and ticks is now being considered for different epidemiological situations.

E1e.1 Support for improved control of tick-borne diseases by national and regional programs

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The development of more sustainable tick-borne disease control methods depends heavily upon the use of immunization against *Theileria parva* and a reduction in the reliance on intensive short-interval acaricide application. The use of immunization and strategic acaricide application is being adopted cautiously by a number of countries within the East African region and ILRAD has been assisting and advising a number of governments, including Zimbabwe, in their moves towards the adoption of these newer control measures.

The distribution and economic losses to theileriosis in Zimbabwe are still poorly understood and information is needed before a decision on disease control policy changes can be made. As part of the study of the epidemiology of theileriosis, 30 isolates from different areas of Zimbabwe have been characterized at ILRAD using a panel of monoclonal antibodies (MAbs) and five DNA probes. Isolates were distinguished as *T. parva* or *T. taurotragi* using the MAbs and a DNA probe for an extrachromosomal element of *T. parva*. Using a *T. parva* Boleni repetitive DNA probe, it was possible to distinguish *T. taurotragi* isolates on the basis of restriction fragment length polymorphisms (RFLPs). *Theileria parva* isolates derived from cattle from widely divergent areas were relatively homogeneous when examined using MAbs or RFLPs using the repetitive or ribosomal DNA probes, but they were different using the telomeric probe. Buffalo-derived isolates were distinguished from cattle parasites by MAbs and RFLPs using these probes. The molecular and antigenic characterization does not support sub-speciation of the *T. parva* parasites found in Zimbabwe. Primers for PCR amplification, DNA probes and monoclonal antibodies were transferred to Zimbabwe during the year and staff from the laboratory in Harare were trained in the use of these reagents.

E1e.2 Studies on the preparation, handling and storage of *Theileria parva* stabilates of sporozoites

Student: E. Kimbita
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The standard method of preparation of stabilates of *Theileria parva* sporozoites involves homogenization of infected *Rhipicephalus appendiculatus* in Eagle's minimum essential medium (MEM) supplemented with 3.5% bovine serum albumin (BSA). Experiments were conducted to compare media for their ability to protect

sporozoites during freezing. Sporozoites were prepared in Eagle's MEM, Iscove's MEM, Leibovitz-15, RPMI 1640, Optimem and buffered lactose peptone, all with 3.5% BSA and 7.5% glycerol. The parasites were then frozen overnight in a –70 °C deep freezer and stored in liquid nitrogen. The sporozoites were thawed and their infectivity for bovine peripheral blood leucocytes was tested by a titration assay *in vitro*. After four weeks, the end-point titres were determined. The lowest end-point titre was obtained with L-15 medium.

In the standard preparation method for stabilates, adult *R. appendiculatus* infected with *T. parva* as nymphs are pre-fed on rabbits for four days before homogenization. It is known that parasite maturation can be induced by holding infected ticks at 37 °C. Investigations were carried out to determine the infectivity of sporozoites from ticks which had been stimulated using heat. Adult ticks infected as nymphs were divided into three groups. One group was incubated at 37 °C and 85% relative humidity for six days, another group was incubated similarly for two days, followed by feeding on a rabbit for three days and the third group was fed on a rabbit for four days. The female ticks from the group which had fed for four days had 100-fold more infective sporozoites than the male ticks fed for the same period. The combination of heat stimulation followed by feeding on rabbits improved the sporozoite infectivity and harvest from male ticks such that the end-point titre was very close to that of sporozoites from female ticks. However, this combined treatment did not have any effect on the infectivity of sporozoites from female ticks. It was not possible to detect infective sporozoites from ticks incubated for six days, although infected acini could be observed histologically. Experiments are in progress to investigate the effects of heat stimulation on ticks immediately after moulting.

E1e.3 The susceptibility of N'Dama cattle to East Coast fever

Scientists: T. Dolan, D. Williams
Technicians: M. Opollo, J. Kiarie

N'Dama cattle have lived in trypanosomiasis endemic areas of West Africa for over 5,000 years and have evolved an effective resistance to the disease. The mechanisms of this resistance, termed trypanotolerance, are not known. It has been claimed that N'Damas are not only resistant to trypanosomiasis but also to other parasitic diseases, such as cowdriosis and helminthiasis, suggesting that the mechanisms of resistance may not be restricted to trypanosomiasis. However, these claims have not been corroborated by published data. The possible introduction of N'Dama cattle into trypanosomiasis challenge areas in other parts of Africa raises the question of their susceptibility to other diseases. East Coast fever is the most important tick-borne disease in East, Central and Southern Africa and it has an overlapping distribution with trypanosomiasis in many countries within the region. An experiment was conducted to test the susceptibility of a group of five N'Dama (raised at ILRAD, after their transfer as embryos from the Gambia) to infection with *Theileria parva*. The N'Damas were infected with an LD100 dose of *T. parva* Muguga 3087 (1.0 ml at 1:20 dilution) together with two susceptible Boran cattle and the disease courses monitored and compared. No differences were observed between the two cattle types

in pre-patent period to detection of schizonts or piroplasms, although fever occurred later in the Borans. The onset and pattern of fall in total leucocytes and lymphocytes were similar. Both groups developed severe clinical disease of a similar character and were treated with buparvaquone on days 16 and 18. At the infective dose studied there was no difference in susceptibility between the N'Dama and Boran cattle. However, the study was not exhaustive and it could be that titration of the infective dose might demonstrate differences in susceptibility. As a preliminary observation there is no evidence of tolerance in N'Dama cattle to East Coast fever.

E2a.1 Sequence analysis of the p67 gene from different stocks of *Theileria parva*

Scientist: V. Nene
Research Associate: E. Gobright
Technician: S. Wanyonyi

The DNA sequence of the p67 gene from the *Theileria parva* Muguga, Marikebuni (bulk), Marikebuni (clone), Ngong and Mariakani parasite stocks has been determined to ascertain the extent of amino acid sequence diversity of the p67 protein in *T. parva* parasites derived from cattle. The genes from *T. parva* (Boleni) and buffalo-derived *T. parva* (7014) have also been sequenced.

Sequence data on several cloned p67 polymerase chain reaction (PCR) products revealed that the amplification procedure introduced errors at a relatively high frequency. These were the only sequence changes detected in the p67 gene from cattle-derived parasites. The p67 gene from buffalo-derived *T. parva* parasites had a 10-fold higher number of nucleotide substitutions and a 129 bp insertion.

To avoid PCR induced errors, direct sequencing strategies were employed using the Promega fmol sequencing kit. The p67 gene of the Marikebuni (bulk) and buffalo-derived parasite were sequenced. There were no differences between the Marikebuni and Muguga gene sequence. In contrast, the buffalo-derived parasite gene has 64 nucleotide substitutions which result in 35 amino acid substitutions, and the presence of the 129 bp insertion was confirmed. Since the latter is in frame it could result in a 43 amino acid sequence insertion. The 129 bp DNA sequence does not have the characteristics of a *T. parva* intron but the presence of the sequence in cDNA remains to be confirmed. The 129 bp DNA sequence may be a valuable marker for studying the epidemiology of cattle- and buffalo-derived parasites.

The current sequence data would suggest that there is very little if any sequence variation of the p67 gene in cattle-derived parasites. Hence, in developing a p67-based sporozoite vaccine, it is reasonable to assume that cross-challenges with cattle-derived parasite stocks will be equivalent to a homologous challenge. In contrast, buffalo-derived parasites contain several predicted amino acid differences in p67. To assess the potential of p67 as a broad spectrum, vaccine antigen candidate it will be essential to conduct cross-immunity trials with buffalo-derived parasites.

E2a.2 Expression of genes encoding the p150 and p104 sporozoite antigens of *Theileria parva*

Scientists: R. Bishop, A. Musoke
Research Associates: R. Skilton, C. Nkonge

A genomic clone encoding the entire open reading frame (ORF) for the p150 antigen of *Theileria parva* has been sequenced and by Southern blot analysis has been found to be parasite-specific. The ORF is 4359 bp long and potentially

encodes a 165-kDa protein which contains a signal sequence. Attempts to clone the full length gene into four different expression vectors, using four different *Escherichia coli* host strains, have not been successful. Recombinants were rare and in any case carried large internal deletions. These difficulties may be due to the high A+T content of sub-regions of the gene. The only successful expression construct encodes about 19% of the C-terminal end of p150 as a Sj26 fusion protein. Large quantities of purified fusion protein have been produced for the production of monoclonal antibodies and for assessment in a diagnostic ELISA test for *T. parva*.

A 5,000 bp genomic clone in pUC19 (pgT-17) encoding the *T. parva* p104 antigen lacks suitable restriction enzyme sites to subclone the full ORF. The ORF was amplified by the polymerase chain reaction (PCR) using primers complementary to the 5′ and 3′ ends of the gene. *Eco*RI sites were included in the primers to facilitate cloning. *Escherichia coli* transformed with a recombinant pGEX-1N vector expressed a near full length fusion protein, as determined by immunoblotting with C16 antiserum. The purified protein will be assessed for its potential in ELISA tests. Fragments of the p104 gene have been inserted into pGEX-1N and these constructs also express fusion proteins as determined by staining of protein gels and by immunoblotting. A PCR product comprising 98% of the ORF lacking the signal sequence is currently being cloned into pGEX-2T.

E2a.3 Standardization of the production of recombinant proteins *in vitro*

Scientists: H. Hirumi, T. Urakawa, V. Nene
Research Associate: K. Hirumi
Technicians: F. Chuma, A. Muthiani

The production of recombinant proteins coded for by the *Autographa californica* nuclear polyhedrosis virus (AcNPV) expression vector, which are expressed upon infection of the *Spodoptera frugiperda* cell line (SF2IAE), has become one of the support functions of the ILRAD cell culture unit. Based on an examination of growth characteristics of the SF2IAE cell line (maximum population density: 2.3 $\times 10^6$ cell/ml) the optimum culture condition (daily seeding density: 3×10^5 cells/ml; yield of cells: 1×10^8 cells in 200 ml/24 hr) in maintaining the cell population has been established. A procedure to increase a cell population from 5×10^6 cells to 5×10^8 cells has been standardized in order to accommodate the semi-large-scale production of recombinant proteins. An adaptation of SF2IAE cells to a medium containing a low concentration of foetal bovine serum (FBS) (10 to 1%) has been made in order to facilitate expressed protein production with a minimum contamination from serum proteins. Further improvement to establish a serum-free system will be continued. An assay system for quantifying plaque forming units (PFU/ml) of AcNPV encoding genes to be expressed, has been standardized in order to achieve the optimum yield of recombinant proteins. Procedures for the cloning and amplification of AcNPVs encoding recombinant genes have been also established *in vitro*.

E2b.1 Stable transfection of *Theileria parva* genes into mammalian cells

Scientist: P. Toye
Technician: J. Nyanjui

The availability of mammalian cells permanently transfected with *Theileria parva* genes is considered to be important to studies aimed at the identification of antigens recognized by immune cytotoxic T-lymphocytes (CTL). As a model for these studies, we have attempted to transfect permanently the cDNA versions of the sporozoite p67 and p104 genes into L cells expressing the bovine class I MHC molecule, KN104. The cDNAs were subcloned into the eukaryotic vector, pcDNA-I-neo, and the recombinant plasmids were transfected into the KN104-expressing L cells by calcium phosphate-mediated gene transfer. Transfected colonies were selected by cultivation in medium containing the antibiotic G418. Neither population of transfectants contained cells reactive with respective antibodies, although Southern blot analysis revealed the presence of the parasite genes in the transfectants. The cells were cloned by limiting dilution, and individual clones were analysed by Northern blotting. In both populations, clones containing RNA corresponding to the respective genes were identified.

E2b.2 Isolation and screening of random schizont cDNA clones

Scientists: V. Nene, R. Bishop, E. Taracha, P. Toye
Research Associate: E. Gobright
Technicians: S. Wanyonyi, J. Gachanja

One approach to identify schizont DNA encoding cytotoxic T-lymphocyte (CTL) epitopes is to screen transfected cells in a direct killing assay using bulk cytotoxic cell lines and/or cytotoxic cells generated in limiting dilution cultures at clonal or near-clonal cell densities. A COS cell system expressing the KN104 haplotype is being explored to serve as target cells for this purpose.

An infected lymphoblast cDNA library in pCDM8 was constructed and may serve as the source of schizont cDNA. Twelve randomly picked colonies were all found to contain inserts which varied in size from about 500 bp to about 1,400 bp indicating a very low level of non-recombinants. Southern blot analysis with total piroplasm genomic DNA gave hybridization signals with three of the 12 clones. As a preliminary screen about 400 colonies were probed with total piroplasm genomic DNA. Fourteen colonies were picked out as potential parasite-specific clones; three did not contain detectable insert DNA and only two of the remaining 11 were positive by Southern blot analysis. Several bovine-specific cDNAs have been isolated by screening with Con A lymphoblast cDNA. The method is being refined to facilitate the isolation of schizont cDNA clones.

E2b.3 Isolation and characterization of naturally processed peptides of *Theileria parva*

Scientists: A. Musoke, D. McKeever, W. Fish, E. Taracha, D. Hunt
Research Associates: C. Nkonge, G. Lamb
Technician: E. Awino

Experiments have been initiated to isolate and characterize processed peptides from class I MHC molecules of *T. parva*-infected cell lines that constitute epitopes for $CD8^+$ T-lymphocytes. *Theileria parva*-infected cells were lysed in detergent and the MHC-peptide complexes were isolated from the lysate using protein A-sepharose coupled to a class I MHC-specific monoclonal antibody. The associated peptides were removed from the MHC molecules by acid elution. Attempts to sensitize uninfected target cells to lysis by *T. parva*-specific CTL with the eluted peptide fraction were unsuccessful. This was attributed to the presence of trace amounts of detergent in the fraction, and the probability that individual specificities were present in low frequencies.

Current efforts are focused on HPLC fractionation of eluted peptides. These fractions will be used in further sensitization experiments and, in addition, their profiles will be compared with those derived from Concanavalin A-stimulated blasts in an effort to identify parasite-specific components.

E2b.4 Identification and characterization of mediators of cell-mediated immunity by phosphorylation with casein kinase II

Scientists: O. ole-MoiYoi, P. Toye
Research Associate: M. Macklin
Technician: J. Osaso

The approaches to the identification of antigens associated with cell-mediated immune (CMI) responses employing binding to, or phosphorylation by, casein kinase II (CKII) include direct chromatographic purification of fractions likely to contain such molecules, and exploitation of immunoprecipitation using antibodies and affinity chromatography. Attempts to purify these proteins by two dimensional gel electrophoresis have not succeeded because of the tendency of the proteins to aggregate giving smears on gels at pIs < 4.0. Purification of CK II from *Theileria parva*-infected lymphocytes or *T. parva* piroplasms yielded insufficient amounts of enzyme. Enzyme has thus been purified from bovine brain by sequential chromatography on phosphocellulose, hydroxylapatite, heparin-agarose and gel filtration. The purification scheme yields homogeneous CKII which gives the typical heterotetrameric subunit pattern of mammalian CKII. This enzyme has now been coupled to activated CH Sepharose for use in direct affinity chromatography of lysates from metabolically-labelled *T. parva* schizonts and infected lymphocytes.

Immunoprecipitation has been employed to identify substrates associated with CK II *in vivo*. Immunoprecipitation of lysates prepared from *T. parva*-infected lymphocytes employing an anti-bovine CKII IgG revealed binding of CK II to the antibody.

Although immunoprecipitates phosphorylated casein and phosvitin in a dose-dependent manner, analysis of kinetic parameters revealed that the enzyme was 75% inhibited by the anti-bovine CKII IgG. The regulatory (β) subunit of CK II did not undergo the expected degree of autophosphorylation. There were no detectable substrates that co-precipitated with the enzyme.

The cDNA of the α (catalytic) subunit of bovine CK II has been sequenced and compared with sequences of CK II from other organisms. The bovine and human cDNA sequences are 95% identical. At the amino acid level, the enzyme exhibits 100% identity to its human homologue. The differences between these two species thus reflect preference in codon usage between cattle and human beings for this gene. Furthermore, comparison of the deduced amino acid sequence of the bovine CK II α with those from organisms as diverse as *Theileria, Zea mays, Caenorhabditis elegans, Drosophila melanogaster* and chicken reveals a remarkable degree of structural conservation, which would, therefore, imply a parallel degree of conservation of the substrates of CK II. The bovine CK II cDNA and that from *T. parva* are being employed to produce fusion proteins for use in affinity selection of CK II substrates.

Other approaches that have been employed in the characterization of CK II substrates in *T. parva*-infected cells include immunoprecipitation using an anti-c-Mil antibody. This antibody revealed that, although c-Mil antigen (71–74 kDa) was present in equal amounts in the G6 IL2-dependent lymphoblasts and in *T. parva*-infected lymphocytes, there were cross-reacting antigens of Mr 80–130 which were substantially increased in the *T. parva*-infected lymphocytes. Similar experiments are in progress employing antibodies or probes for pRB105, p53, large T antigen of SV-40 and similar nuclear transcription-regulating substrates of CK-II.

E2b.5 Generation of CD4+ *Theileria parva*-specific T-cell clones from immune cattle

Scientists: D. Grab, D. McKeever, A. Musoke
Research Associates: G. Lamb, C. Nkonge
Technician: Y. Verjee

There is growing evidence that the induction of bovine cytotoxic lymphocyte (CTL) responses is dependent on the input of CD4+ T-cell subsets. In addition, this population is likely to be involved in alternative protective mechanisms, as inducer or effector cells. Previous reports have described the generation of bovine CD4+ T-cell clones specific for a component of the high-speed supernatant fraction of *Theileria parva*-infected cell lysates. These clones are no longer available, but their target antigen in the high speed supernatant fraction (HSS) of *T. parva* has been resolved to a polypeptide of Mr 24 kDa. For these reasons, efforts have been made to generate fresh CD4+ T-cell clones specific for *T. parva*-infected cells and HSS. Experiments have all been based on the use of peripheral blood monocytes (PBM) derived from E98, an animal whose MHC phenotype and *T. parva*-immune status have been well characterized. Despite the use of a number of culture and cloning conditions, it was not possible to demonstrate specific CD4+ T-cell activity directed at HSS components. A number of CD4+ T-cell clones were however generated against *T. parva*-in-

fected lines. A reasonable conclusion is that E98 PBM do contain CD4+ T cells specific for *T. parva*-infected cells, but that these specificities are not present in HSS. Thus, further studies in this area will include an assessment of the frequency of T-cell reactivity for HSS components in a number of cattle.

A fusion has been performed using a mouse immunized with nitrocellulose floccules generated from the 24-kDa band of a Western blot of the hydroxylapatite fraction of HSS. The pre-fusion serum was seen to identify a band of approximately 30-kDa on a Western blot of a *T. parva*-infected cell line. Clones from this fusion are currently being screened for specificity. These monoclonal antibodies will constitute useful screening reagents for the identification of the gene that encodes this antigen.

E2c.1 Cloning and expression of the gene encoding the 32-kDa antigen of *Theileria mutans*

Scientists: S. Morzaria, V. Nene, A. Musoke
Research Associates: R. Skilton, C. Nkonge

Full length inserts of 32-kDa cDNA homologues, clones 9 and 23, were excised from Bluescript SK- constructs using *Sma*I and *Cla*I. Purified inserts were blunt ended and single 3′ 'A' additions were made using dATP and Taq polymerase prior to ligating into *Stu*I digested pMG1 T-vector. *Escherichia coli* strain MM294 (cI^+) was transformed with the ligation mixture. Recombinants containing inserts in the correct orientation were identified and the DNA sequence at the junction of pMG1 and insert were determined to ensure that the constructs were in frame. Recombinant plasmids were transferred into the *E. coli* expression host AR 58 (cI857) and expression induced by heat shock at 42 °C. Western blots of total cell lysates at 0, 1, 2 and 3 hours after heat shock were probed with bovine serum D721. Steer D721 had been hyperimmunized with lysates of *Theileria mutans* piroplasms and its serum reacts strongly with the 32-kDa antigen in blots of piroplasm lysates. Heat shock appears to induce D721 reactive bands up to the size expected for a NS1-32-kDa fusion protein. These bands are not induced in cells containing non-recombinant pMG1. These results indicate that the cloned cDNA expresses epitopes recognized by the D721 antiserum. The fusion protein will be evaluated as a vaccine against *T. mutans* and as a target in antibody ELISAs for detection of infection of cattle with *T. mutans*.

E3a.1 Evaluation of a novel adjuvant for delivery of recombinant p67

Scientists: A. Musoke, D. McKeever, V. Nene, S. Morzaria, J. Gerber*
Research Associates: C. Nkonge, J. Katende, P. Spooner
Technicians: L. Gichuru, J. Ngugi, T. Ndolo, D. Lugo, J. Kiarie

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Expression of p67, the major surface antigen of *Theileria parva* sporozoites, in *Escherichia coli* using the pMG1 expression system has been described in a previous report. The recombinant protein, NS1-p67, was shown to give rise to protective immunity in approximately 70% of immunized animals when administered in saponin. During the year, an adjuvant formulation developed by SmithKline Beecham Animal Health (SKB) was evaluated for use in p67 immunizations.

The NS1-p67 recombinant protein was purified by scientists at SKB and administered with the adjuvant to a group of five cattle. The animals received 400 μg of NS1-p67 as a priming dose and were given three identical booster inoculations at monthly intervals. The animals were challenged with an LD68 of *T. parva* stabilate 3087 three weeks after the last booster. Immune responses were analysed in detail in terms of responding antibody isotypes and T-cell responses.

All five immunized animals showed no reaction to challenge, while the majority of the controls suffered severe clinical disease. One of the control animals resolved the challenge infection.

Specific serum antibody titres were similar in all animals, both in quantity and neutralizing capacity. A classical isotype response was observed with IgM antibodies appearing first, followed by IgG_1 and IgG_2. The response was dominated by the IgG_1 subclass. Interestingly, all isotypes appeared after primary immunization suggesting that this adjuvant produces a good depot effect. This is supported by the observation that titres remained high throughout the immunization period, in contrast with animals immunized previously with NS1-p67 formulated in saponin.

To screen for sub-clinical infections not detected by microscopy or serology, lymph node biopsies and blood samples from the non-reacting animals and those that showed parasitosis were analysed for the presence of parasites by the polymerase chain reaction (PCR). Primers derived from p67 DNA and from *T. parva* repetitive DNA sequences were used. Only the animals that had showed parasitosis yielded detectable PCR products, suggesting that the parasites were eliminated from protected animals before the establishment of a schizont parasitosis.

As in previous experiments, p67 specific T-cell activity was not detectable in the NS1-p67 immunized cattle, although these responses were evident in assays performed after challenge. It appears from these results that the adjuvant augments the induction of protective immune responses to recombinant p67 in cattle. Further studies will focus on the determination of the minimum quantity of NS1-p67 required to induce protection against both homologous and heterologous challenge.

E3a.2 Evaluation of baculovirus-derived p67 as an immunogen

Scientists: A. Musoke, V. Nene, D. McKeever
Research Associates: C. Nkonge, J. Katende, P. Spooner
Technicians: L. Gichuru, J. Ngugi, D. Lugo

As reported previously, the gene encoding p67 has also been expressed in the baculovirus vector system. Unlike bacterial expression systems, the insect cells carry out post-translational modifications on the introduced gene product. This may explain why, to date, this recombinant p67 product (BEVp67) is the only recombinant protein that has shown reactivity with monoclonal antibody TpM12, which can neutralize the infectivity of live *T. parva* sporozoites.

The BEVp67 antigen was prepared from insect cell-cultures on day 4 of infection by lysis in 1% NP-40. Two groups of three cattle were immunized, one with BEVp67 in 3% saponin and the other with the antigen in Freund's complete/incomplete adjuvant. The cattle were each given five inoculations of approximately 140–280 μg of BEVp67. Ten days after the last boost, animals were challenged with an LD68 of *T. parva* stabilate 3087 and immune responses were analysed as described in abstract E3a.1.

On challenge, two animals in each group showed no clinical disease while the remaining two reacted severely. Specific antibody responses were similar in both groups, with ELISA titres ranging from 1:50,000 to 1:150,000, and neutralization titres between 1/300 and 1/500. Parasite DNA was not detectable in non-reactor animals by polymerase chain reaction analysis. As with the animals immunized with NS1-p67, p67-specific T-cell activity was not detectable in these animals up to the time of challenge.

E3a.3 Epitope mapping of the p67 molecule of *Theileria parva*

Scientists: D. McKeever, A. Musoke, V. Nene
Technicians: E. Awino, J. Ngugi

For reasons that are not clear, in all cattle immunized with the recombinant sporozoite protein of *T. parva*, NS1-p67, p67-specific T-cell activity has not been detected up to the time of challenge, in spite of the presence of high antibody titres. These cellular responses can however be detected after challenge of the animals with sporozoites.

In an attempt to resolve this anomaly, 18 p67-specific T-cell clones have been generated from animals immunized with the native antigen. All of the clones react with the native antigen, but only six recognize the NS1-p67 recombinant molecule. These results suggest that an important epitope of the native molecule is not represented on the recombinant antigen. It is not clear whether this is a reflection of structural differences between the two antigens, or the result of improper processing of the recombinant molecule in antigen presenting cells. To address this question, and to define important neutralizing epitopes, the synthesis of overlapping peptides covering the entire sequence of the molecule has been commissioned. These reagents will be used in ELISA analyses and T-cell proliferative assays.

E3b.1 Generation of improved constructs for appropriate expression of parasite genes incorporated in recombinant vaccinia viruses

Scientists: D. McKeever, M. Juarrero
Research Associate: R. Kennedy
Technician: W. Mwangi

Results of a number of experiments have indicated that the currently available construct for the expression of *Theileria parva* p67 in vaccinia virus-infected cells is unsatisfactory. The antigen does not appear to be expressed on the cell surface and instead seems to target to a vesicular compartment. This may in part explain the disappointing antibody titres generated in immunized animals, and the failure to detect cytotoxic T lymphocyte (CTL) responses against the antigen. During the past year, an effort has been made to generate new constructs with the purpose of favouring antigen processing mechanisms for the generation of either antibody responses or CTL. For the induction of antibody, two strategies have been employed to give rise to surface expression of the antigen. The first of these involved the construction of a chimaeric gene incorporating codons for the first 644 residues of p67 fused to those of the carboxyl 28 residues of the *Trypanosoma brucei* ILTat 1.3 variable surface glycoprotein gene cDNA. The purpose of this was to give rise to a phosphoinositol-linked membrane anchor on the molecule that might target it for surface expression. This chimaera has now been constructed in the pUC19 plasmid, and is currently being subcloned into the vaccinia shuttle vector p1114. The second strategy to obtain surface expression of p67 is the construction of a p67-chimaera with the transmembrane and cytoplasmic portions of the KN104 bovine class I MHC gene.

E3b.2 Effects of serpin gene deletions on immunogenicity of recombinant vaccinia virus constructs

Scientists: M. Juarrero, D. McKeever
Research Associate: R. Kennedy
Technicians: W. Mwangi, F. Mbwika

We have previously reported that recombinant vaccinia viruses expressing the p67 surface sporozoite antigen of *Theileria parva* induce poor immune responses in cattle. Possible reasons for this are the lack of p67 surface expression on infected cells, poor processing of parasite antigens by infected eukaryotic cells, poor activation of specific immune responses by the virus vector, or a combination of all of these factors.

Vaccinia virus expresses serine protease inhibitors (serpins) during the early phase of its infection cycle, possibly as a strategy to evade the host's immune response. Serine proteases are believed to be important for the activation of host immune responses against viral infections; they participate in the biosynthesis of chemo-attractant substances released at the site of infection and they are believed to be involved in the activity of cytotoxic T lymphocytes (CTLs). Inactivation of serpin genes has been reported to enhance the capacity of recombinant vaccinia viruses to

induce host immune responses. Increased numbers of leukocytes are observed at the site of infection, and both antibody and CTL responses are enhanced.

To evaluate the effect of serpin gene inactivation on bovine immune responses to recombinant vaccinia viruses, plasmids containing each of two vaccinia serpin genes (SPI, SPII) have been obtained. These have been used to generate serpin-deleted derivatives of the p67-recombinant virus Wyp67. Recombinants with deletions in either or both serpin genes are now available, and these will be evaluated for enhanced immunogenicity *in vivo* and *in vitro*.

E3c.1 Protection of immune cattle against challenge with *Theileria parva* in the absence of detectable cytotoxic lymphocyte responses

Scientists: D. McKeever, E. Taracha
Research Associate: N. MacHugh
Technician: E. Awino

A number of observations indicate that a proportion of immune cattle are protected against challenge with *Theileria parva* through the use of effector mechanisms other than parasite-specific cytotoxic lymphocyte (CTL) activity. A series of five lymphatic cannulation experiments were performed to consolidate these observations and to investigate the nature of the alternative responses. With one exception, these experiments involved the transfer of lymphocyte populations between immune and naive twin calves during the peak of the donor response to challenge. In only one of these animals were CTL responses observed during the challenge infection and, on this occasion, transfer of the $CD8^+$ fraction of responding lymph to the naive twin was observed to confer protection. In all other instances, CTL responses were not detected during the course of the challenge and, when transferred, the $CD8^+$ fraction did not confer protection on the corresponding naive twin.

In one experiment, although the donor animal had detectable CTL precursors in its circulation prior to challenge, these cells were not observed to respond during the challenge. On this occasion the $CD8^+$ fraction of responding lymph was transferred to the naive twin during the peak of the immune response. The recipient resolved the challenge. This animal was later rechallenged and cannulated in an attempt to assess the nature of its immunity. Again, although CTL precursors were present in the circulation at low frequency at the time of challenge, no cellular response was detected in the draining lymph during the course of infection. Lymph node biopsy samples taken at various time points after challenge suggested that considerable cellular activity was present in the node, but these samples did not contain detectable parasite-specific activity.

In conjunction with the results of analyses of immune responses of young cattle to immunization and challenge [see abstract E3c.5], these observations provide further evidence that a significant proportion of cattle do not respond to infection-and-treatment immunization by generating parasite-specific CTL.

E3c.2 Inductive requirements of bovine cytotoxic lymphocytes

Scientists: E. Taracha, D. McKeever
Technician: E. Awino

To define the factors that are important for the generation of primary and secondary responses by bovine $CD8^+$ cytotoxic lymphocytes (CTL), highly purified (96–99%) populations of $CD4^+$ and $CD8^+$ T cells have been used in co-culture experiments with *Theileria parva*-infected lymphoblasts. The results of these analyses have indicated that $CD4^+$ T cells are important for the generation of primary and memory $CD8^+$ T-cell responses.

Experiments utilizing combinations of $CD4^+$ and $CD8^+$ T cells derived from *T. parva*-immune animals, as well as those of immune $CD4^+$ and non-immune $CD8^+$ T cells, yielded antigen-specific $CD8^+$ CTL activity comparable to that of unfractionated immune cells. This activity was significantly higher than that generated in cultures containing only $CD4^+$ or $CD8^+$ T cells as responders. Activity in cultures of non-immune $CD4^+$ with immune $CD8^+$ T cells was mid way between these two. Co-cultures of $CD4^+$ T cells derived from an animal primed with trypanosomal variable surface glycoprotein (VSG) with MHC-matched *T. parva*-immune $CD8^+$ T cells also yielded parasite-specific CTL. This activity was nearly two times higher when monocytes were included as antigen-presenting cells (APC) for the VSG (four-cell cluster) than when presentation was by the autologous *T. parva*-infected cells of the culture (three-cell cluster). In these experiments, co-cultures of VSG-primed $CD4^+$ and *T. parva*-immune $CD8^+$ T cells yielded significant CTL activity even in the absence of VSG. This is believed to be a reflection of autologous mixed lymphocyte reactions in the culture. However, cultures incorporating VSG-primed $CD4^+$ T cells and non-immune $CD8^+$ T cells in the presence of VSG did not yield measurable CTL activity, even with added monocytes.

It is evident from these results that *T. parva*-immune and naive $CD8^+$ T cells both require help from $CD4^+$ T cells to generate CTL activity. This help is more effective with antigen-specific $CD4^+$ T cells, but it is probable that bystander activity by $CD4^+$ T cells also has an effect on immune but not naive $CD8^+$ cells. Further work is planned to define the signals that $CD4^+$ T cells provide to $CD8^+$ T cells using *T. parva*-infected cells as APC as well as other APC presenting defined soluble antigens to T cells.

E3c.3 Identification of monoclonal antibodies that define functional T-cell subsets of cattle

Scientists: N. MacHugh, C. Howard*, G. Bembridge*, D. McKeever
Technician: E. Owino

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The identification of the cell populations responsible for effector and memory functions within the bovine immune system is essential to the study of the inductive requirements of primary and secondary immune responses in cattle. By performing a series of fusions and selecting antibodies on the basis of their ability to subset CD4 and CD8 cells, we have attempted to generate monoclonal antibodies that differentiate memory from naive cells within these subpopulations. A number of antibodies were selected on this basis and one (ILA-150) was characterized further. The specificities of the remaining antibodies have yet to be determined.

The monoclonal antibody (MAb) ILA-150 recognizes a 180-kDa glycoprotein present on the surface of a subset of bovine T cells, $\gamma\delta$ T cells and monocytes, but is absent from the surface of B cells. The T-cell population recognized includes both $CD4^+$ and $CD8^+$ cells. Based on the tissue distribution and the relative molecular mass of the antigen recognized, we believe that ILA-150 is specific for the bovine homologue of CD45RO.

To investigate the effector function of the ILA-150 defined T-cell subsets, $CD4^+$ T cells from an animal immunized with the variable surface glycoprotein (VSG) of *Trypanosoma brucei* were sorted as $CD4^+ILA\text{-}150^+$ or $CD4^+ILA\text{-}150^-$. After incubation with VSG in the presence of antigen presenting cells, cellular proliferation was restricted to the $ILA\text{-}150^+$ population. These results suggest that ILA-150 is a possible marker for $CD4^+$ memory T cells or that it identifies TH1 or TH2 subsets of helper T cells.

E3c.4 Analysis of the clonality of cytotoxic T lymphocyte responses in *Theileria parva*-immune cattle

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

It has been observed in a number of *Theileria parva*-immune cattle that, at the peak of their immune response to challenge, the lymph leaving the node under challenge contains parasite-specific cytotoxic T lymphocytes (CTL) at frequencies as high as 1:30. The kinetics of the challenge infection in these animals suggest that these cells are responsible for clearance of the parasite, and this is supported by the results of two cell transfer experiments between immune and naive twin calves. We have attempted to evaluate the clonality of these responses by analysis of the T-cell receptor (TCR) variable gene usage in T-cell clones derived from responding lymph. Four such clones have so far been analysed, along with two clones derived from peripheral blood. Primers for the polymerase chain reaction (PCR) were designed on the basis of a study of previously determined TCR gene sequences, and these were used to amplify variable and junctional segments from cDNA derived from the clones. Amplified products were then directly sequenced using the Fentomole sequencing kit. Sequences are now available from α and β TCR genes from three of the clones, while α chain sequences have been determined for the remaining two. In the case of two of the clones, interpretation of the sequence data has been hindered by an apparent cross-contamination of α chain products. These reagents will be useful for the design of probes for *in situ* hybridization studies of bulk responding lymphocyte populations.

E3c.5 Immune competence of young calves

Scientists: J. Scheerlinck, T. Van de Putte*, S. Morzaria, D. McKeever
Research Associate: N. MacHugh
Technician: D. Ngugi

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Because calves are the main target for vaccination against *Theileria parva*, the ability of young calves to generate cytotoxic T lymphocytes (CTL) responses was studied. This mechanism is thought to be of major importance in the protection of adult

animals against the parasite. Peripheral blood mononuclear cells (PBMC) from eight young cattle (aged from 1 to 20 weeks) were characterized at different time intervals. It was observed that the proportion of $CD4^+$, $CD8^+$ and $CD2^+$ T cells and monocytes remain essentially unchanged over a period of four months. However a significant decline in the percentage of $WC1^+$ cells was observed and this was associated with a reduction in the numbers of $CD3^+$ T cells. A corresponding increase in the proportion of IgM^+ cells was also observed.

Peripheral blood mononuclear cells from a group of six young cattle were assessed for the capacity to generate a mixed lymphocyte reaction response against irradiated cells from mismatched adult animals. It was observed that cells stimulated in this way failed to generate CTL activity that was measurable in a classical four-hour assay. At about 12 weeks of age the animals were immunized with a clone of *T. parva* (Marikebuni) using the infection-and-treatment method. After immunization, PBMC from four out of five animals failed to show CTL activity against autologous *T. parva*-infected cells. Precursor cells could be demonstrated in some animals by limiting dilution analysis, although at low frequencies. Three months after immunization the animals were challenged with the cloned parasite. All six calves were protected, while the two control animals suffered severe clinical reactions. On days 7, 9 and 11 following challenge, time periods shown previously in adult animals to be optimal for the detection of CTL, none of five animals had detectable CTL against autologous *T. parva*-infected cells.

These results suggest that a CTL-independent mechanism might contribute to recovery from challenge in animals immunized at a young age. However, further investigations are required to exclude the possibility that CTL home to different lymphoid tissues in young animals and are not detectable in peripheral blood.

E3c.6 Novel adjuvant formulations as safe and efficient non-recombinant antigen-delivery vehicles

Student: A. Diate
Supervisor: D. McKeever

Thirty Boran calves were divided into six groups of five animals each. Four groups were used to test antibody and T-cell responses to a *Trypanosoma brucei* variant surface glycoprotein (VSG) delivered either in the Ribi adjuvant system, Titer Max, immunostimulating complex (ISCOM) or Freund's complete adjuvant formulations. One of the remaining groups was used to assess responses to immunization with NS1-p67 formulated in ISCOMs, and the other to assess the modulation of antigen-presenting cell function at the site of deposition of the adjuvant.

To date, the ability of the different systems to prime the immune system has been assessed only in terms of the response of T cells. Results clearly show a superiority of the ISCOM system over the others. Priming of the immune system is more rapid and stimulation indices are higher. In addition, less adjuvant is required for inoculation and the reactions at the site of inoculation are less severe. Sera collected on the days of T-cell assay have been stored at –70 °C for further analysis by ELISA.

Because of the encouraging results seen with VSG-ISCOMS, five Boran calves were immunized with an ISCOM preparation incorporating purified NS1-p67. All animals received 100 µg of antigen in 27.5 µl ISCOM preparation. Results showed only weak T-cell responses between 7 and 14 days after immunization. Booster immunization performed on day 21 failed to provoke any detectable T-cell responses to the native antigen. Similar observations have been made in cattle immunized with this antigen in different formulations. However, neutralization assays performed on sera from the NS1- p67 ISCOM-immunized animals contained only low titres on days 21 after primary and secondary immunization. The latter result may be due to the effect of detergents used in the generation of the ISCOMs as similar observations have been made with certain viral antigens, and have been attributed to denaturation of neutralizing epitopes by these detergents.

TRYPANOSOMIASIS

T1a.1 Maintenance of production colonies of different *Glossina* species

Scientist: S. Moloo
Technicians: N. Kuria, M. Owino, A. Kafwa

The ILRAD 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.

Five tsetse breeding colonies were maintained at ILRAD in 1992. These were *Glossina morsitans centralis* which originated from Singida, Tanzania; *G. pallidipes* from Shimba Hills, Coast Province, Kenya; *G. pallidipes* from Nguruman, Rift Valley Province, Kenya; *G. brevipalpis* from Kibwezi Forest and *G. longipennis* from Nguruman, both in Kenya. In February 1992, the colony of *G. palpalis gambiensis* which originated from Central African Republic was terminated. All five breeding colonies were maintained at 25 °C and were fed five days a week on the ears of half-lop rabbits. *G. m. centralis* and *G. longipennis* were kept at 70% relative humidity, and all the others at 80%. The mean numbers of breeding females of *G. m. centralis*, *G. pallidipes* (Shimba Hills), *G. pallidipes* (Nguruman), *G. brevipalpis* and *G. longipennis* colonies were 10,319, 2,738, 2,732, 1,237 and 1,065; they produced 308,405 (mean weight 34.7 mg), 70,461 (42.0 mg), 59,937 (41.7 mg), 34,983 (78.7 mg) and 25,103 (76.7 mg) puparia, respectively. A colony of *G. fuscipes fuscipes* was initiated in January 1992 with 500 wild puparia from Mbita, Rusinga Island, Kenya. This colony is at the expanding phase and as at the end of August 1992, it had 254 breeding females and produced 1,781 pupae (mean weight 23.5 mg). The five production colonies provided all the tsetse required for trypanosomiasis research conducted at ILRAD in 1992 and also provided research groups in Kenya and abroad with tsetse puparia.

T1a.2 A comparison of the colony performance of *Glossina pallidipes* originating from two allopatric populations in Kenya

Scientist: S. Moloo
Technicians: N. Kuria, M. Owino, A. Kafwa

In October 1986, 88 wild female *G. pallidipes* were caught in Shimba Hills, Coast Province, and in January 1989, 551 *G. pallidipes* were caught in Nguruman, Rift Valley Province, Kenya. These tsetse were put in Geigy-20 cages, 20 tsetse per cage, kept in a sealed box and transported to ILRAD. Each cage consisted of 15 × 8.5 × 5 cm stainless steel wire frame, with a closed end fitted with a cork. The cage was covered with black Terylene netting of 3 mm mesh. The tsetse were kept in a climate controlled room at a temperature of 25 ± 0.5 °C, relative humidity of 80–85%, and 12 hr indirect, dim tungsten light and 12 hr darkness. They were fed on the ears of lop-eared rabbits

daily except at weekends. The field-caught *G. pallidipes* from Shimba Hills and Nguruman produced 119 pupae (mean weight 39.4 ± 0.7 mg) and 447 pupae (mean weight 33.6 ± 0.5 mg), respectively. The adults which emerged from these pupae formed the parental stocks of the two *G. pallidipes* colonies. Standard techniques were used to rear the two colonies, except for the following. For mating, virgin females seven days old, 20 in each cage covered with black Terylene netting, are fed on rabbits' ears and then 22 recently fed 10- to 15-day-old males are introduced into each cage. Mating occurs under indirect dim tungsten illumination, the two sexes being kept together and allowed to feed as above, and seven days later all the males are removed. Female flies are maintained for 90 days and on day 91 post-emergence the surviving flies are killed.

For the 12-month period from September 1991 to August 1992, the mean mated female stocks of the two *G. pallidipes* colonies of Shimba Hills and Nguruman origin were 2,738 and 2,732, respectively. Survival of both these colonies was excellent in that more than 60% survived for 72 days, i.e. eight age-group periods of nine days each. Although the fecundity of both of the *G. pallidipes* colonies was good, that of the Shimba Hills-derived population was better ({X} ± S.E = 0.63 ± 0.01) than that of the Nguruman origin (0.54 ± 0.01). The difference is highly significant ($t = 9.0$, $df = 18$, $P < 0.001$). The insemination rate of the two colonies was 100%. The mean pupal weights of the *G. pallidipes* colonies of Shimba Hills and Nguruman origin were 42.0 ± 0.3 mg and 41.7 ± 0.2 mg; the mean emergence rates were 91.2 ± 0.4% and 91.8 ± 0.3%; and the mean female mortalities were 0.83 ± 0.04% and 0.83 ± 0.05% per day, respectively. Thus, the overall performance of these two *G. pallidipes* colonies originating from the two allopatric populations in Kenya was good and the colonies have been producing adequate surplus for research on their vector competence for pathogenic *Trypanosoma* species.

T1a.3 A comparison of vector competence of two allopatric populations of *Glossina pallidipes* for *Trypanosoma simiae*

Scientists: S. Moloo, E. Zweygarth*
Technicians: C. Sabwa, J. Mulati

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

Teneral male and female *G. pallidipes* from the colony which originated from Nguruman, Rift Valley Province, Kenya, or Shimba Hills, Coast Province, Kenya, were fed simultaneously on Large White pigs infected with *T. simiae* stock CP 11 isolated from *G. austeni* in Shimba Hills. Thereafter, the tsetse were maintained on goats, and on day 30 the surviving tsetse were dissected. Infection rates of five additional groups of teneral tsetse from the two colonies were similarly determined. Male *G. pallidipes* ({X} ± S.E. = 7.6 ± 2.3%) from Nguruman were significantly more susceptible than females (3.0 ± 1.3%; $t = 4.643$, $df = 5$, $P < 0.05$). In contrast, although male *G. pallidipes* from Shimba Hills (2.8 ± 2.3%) showed a higher mature infection rate than females (1.3 ± 0.7%), the difference was not significant ($t = 0.923$, $df = 5$, $P > 0.05$). When data for the two sexes were considered collectively, *G. pallidipes* from the

Nguruman colony (5.3 ± 1.8%) were significantly more susceptible than those from the Shimba Hills colony (2.0 ± 1.4%) to infection with *T. simiae* CP 11 ($t = 5.517$, $df = 5$, $P < 0.01$). Thus, the two allopatric populations of *G. pallidipes* probably differ with regard to the epidemiology of *simiae*-trypanosomiasis in the two areas of Kenya.

T1a.4 Virulence of *Trypanosoma simiae* in pigs infected by different *Glossina* species

Scientists: S. Moloo, E. Zweygarth*
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It was reported that when *T. simiae* stocks are transmitted to pigs by *G. brevipalpis*, the resultant disease is hyperacute whereas the transmission of the same stock to pigs by infected *G. pallidipes* causes chronic disease. A study was carried out to determine if indeed the infecting *Glossina* species determines virulence of *T. simiae* in pigs. Two stocks of *T. simiae* which had been isolated in Muhaka, Coast Province, Kenya, were used. *T. simiae* stock CP 11 was isolated from *G. austeni* and CP 813 from *G. pallidipes*. Both these stocks cause hyperacute disease in pigs. Teneral *G. brevipalpis* and *G. pallidipes* were fed on Large White pigs infected with *T. simiae* stock CP 11 or stock CP 813. Thereafter, these tsetse were maintained on goats and on day 28 the surviving tsetse were induced to probe onto slides warmed to 37 °C to identify those with mature trypanosome infections. Twelve *G. brevipalpis* (8.3%) and four *G. pallidipes* (3.0%) showed *T. simiae* CP 11 metacyclics whilst nine *G. brevipalpis* (5.1%) and seven *G. pallidipes* (6.9%) showed CP 813 metacyclics in their salivary probes. Three *G. brevipalpis* and three *G. pallidipes* infected with *T. simiae* CP 11 were fed singly on six pigs; and two *G. brevipalpis* and two *G. pallidipes* infected with CP 813 were similarly fed on four different pigs. The prepatent period in these ten pigs ranged from four to eight days, and all ten infected animals died within one to four days of patent infection. Thus, the virulence of *T. simiae* in pigs is not determined by the species of *Glossina* transmitting the parasite.

T1a.5 African buffalo, N'Dama and Boran cattle as reservoirs of *Trypanosoma vivax* for *Glossina*

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Teneral *Glossina morsitans centralis* were fed on the flanks of African buffalo, N'Dama or Boran cattle infected with *Trypanosoma vivax* stock IL 2337. The infected

tsetse were maintained on goats and on day 25 after the infected feed, the surviving tsetse were dissected to determine the infection rates. The mean mature infection rates (% ± S.E.) in the tsetse fed on buffalo, N'Dama and Boran cattle were 34.3 ± 9.9, 33.7 ± 13.4 and 58.9 ± 7.1, respectively. Logistic regression analysis indicated that infection rates in the labrum and hypopharynx of the tsetse were significantly lower when fed on the infected buffalo or N'Dama than Boran cattle. Similarly, the risk of infection was significantly lower in male than female tsetse. When teneral *G. m. centralis, G. pallidipes, G. p. gambiensis, G. brevipalpis* and *G. longipennis* were fed simultaneously on either the buffalo cow, the N'Dama bull or the Boran steer infected with *T. vivax* IL 2337, the mature infection rates were higher in the two *morsitans* group tsetse than the two *fusca* group tsetse, whilst *G. p. gambiensis* was relatively refractory to the infection, irrespective of the host species on which they fed. Logistic regression analysis indicated that the infection rates in the labrum and hypopharynx were significantly different amongst the five tsetse species for each of the three infected host animals. Nevertheless, the trypanotolerant African buffalo and N'Dama cattle may serve as reservoirs of *T. vivax* infection as can trypanosusceptible Boran cattle.

T1a.6 Axenic cultivation of *Trypanosoma simiae* bloodstream trypomastigotes *in vitro*

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Cultures of the *Trypanosoma simiae* stock CP 813 which was isolated from wild *Glossina pallidipes* caught in Muhaka Forest, Coast Province, Kenya, were initiated with tsetse-derived metacyclics. Initially they were propagated as trypomastigote forms at 35 °C in the presence of bovine endothelial cells. They were subsequently adapted to axenic culture conditions. The medium consisted of Eagle's minimum essential medium supplemented with 20% heat-inactivated swine serum, 1% of MEM non-essential amino acids, 100 IU/ml penicillin, 100 µl/ml streptomycin, 0.2 mM adenosine, 0.1 mM 2-mercaptoethanol, 20 µM 2, 9-dimethyl-4, 7-diphenyl-1, 10-phenanthroline disulphonic acid and 1 mM L-cysteine. Cultured trypanosomes were morphologically similar to bloodstream trypomastigotes found *in vivo* and retained their infectivity and some degree of virulence for a domestic pig.

T1b.1 Identification, cloning and characterization of a *Trypanosoma brucei* species-specific antigen

Scientists: R. Masake, O. ole-MoiYoi, R. Pelle
Research Associate: S. Minja
Technicians: J. Makau, J. Njuguna

An antigen-detection ELISA has been developed for detecting a *Trypanosoma brucei* species-specific antigen in the blood of infected animals. This test is based on an anti-*T. brucei* species-specific monoclonal antibody, TR7. TR7 reacts with an invariant surface membrane antigen, which is unaffected by periodate digestion. On SDS-PAGE, TR7 recognizes a 32-kDa antigen in bloodstream form trypanosomes but reacts with variably-sized (18, 26, 28, 30 and 32 kDa) proteins in procyclics of *T. brucei*. These proteins were eluted from SDS-PAGE gels, emulsified in Freund's complete adjuvant and thereafter inoculated into rabbits. The rabbits were boosted several times with the same antigen without adjuvant. Hyperimmune serum was finally obtained, absorbed with *Escherichia coli* λgt11.2/Y1089 lysate and employed in the screening of a *T. brucei* cDNA expression library. Clones reacting with the hyperimmune serum were further analysed by the technique of antibody select. The antibodies bound to the clone were eluted and allowed to react with lysates of *T. brucei* bloodstream form parasites after the proteins had been separated on SDS-PAGE. A fairly diffuse zone of reaction was observed at 32 kDa. The *T. brucei* DNA insert has been prepared in bulk and will be transferred to two different vectors for sequencing and production of fusion proteins.

T1b.2 Cloning and characterizing the gene encoding the diagnostic antigen for *Trypanosoma congolense*

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A polyvalent antibody, raised in rabbits immunized with a gel-purified preparation of a 38/40-kDa protein doublet, was used to screen a λgt11 expression library. Nine clones that reacted with the antibody were identified and have been further analysed. The inserts from these clones all hybridized to each other. The clone with the largest insert (lcNTP-1600) was selected for further characterization. The authenticity of this clone, as well as that of the other eight clones, was verified by an 'antibody select' procedure using the expressed proteins. The antibodies, which were eluted from nitrocellulose-bound proteins encoded by the clones, recognized the 38/40-kDa polypeptides of *T. congolense* on Western blots. The genes were polymorphic in the Kilifi savannah and West African riverine and forest subtypes of *T. congolense*. There appeared, therefore, to be a multicopy family of genes

encoding the diagnostic protein. The gene(s) were not detectable in *T. simiae* DNA, suggesting that the diagnostic antigen was specific to *T. congolense* rather than being *Nannomonas*-specific. Southern blot analysis of restriction enzyme digests of the *T. congolense* DNA showed that the gene was conserved and also specific to this species, as no hybridization occurred with DNA digests of *T. vivax* or *T. brucei*. The transcript size on Northern blot analysis indicated that a full length transcript would be 1.8 kb. There appeared to be an equal amount of the transcript in all the life cycle stages of *T. congolense*. However, the protein was not expressed in the procyclic stage of the parasite.

The 1.6 kb cDNA was released from the recombinant clone, lcNTP-1600, with *EcoR*I and cloned into the same enzyme-site of pUR-291. A fusion protein with β-galactosidase was obtained and tested in the *T. congolense* ELISA system. An indirect ELISA assay revealed the reactivity of the antigen with the *T. congolense* antibodies. However, after purification, the fusion protein resolved on SDS-PAGE did not react with various samples of *T. congolense*-infected sera on Western blots. The failure of the fusion protein to react with these antibodies on Western blots may be due to steric effects of β-galactosidase. A polyvalent antibody to the fusion protein was produced in rabbits and was specific to the 38/40-kDa peptide antigens of *T. congolense*. The monospecific, polyvalent antibody was employed in a sandwich antigen-detection ELISA, and had a substantially higher sensitivity than was observed with the original monoclonal antibody. The gene is currently being sequenced and characterized.

T1b.3 Production of trypanosome genus-specific and bovine erythrocyte-specific monoclonal antibodies

Scientists: R. Masake, E. Authié
Research Associate: S. Minja
Technicians: J. Makau, J. Njuguna

An antigen-trapping enzyme linked immunosorbent assay was developed for detection of trypanosome infection in livestock. The monoclonal antibodies (MAbs) employed in trapping trypanosome antigens are species-specific. This has been very useful in the identification of trypanosome species responsible for disease in different places. To get a complete picture of the disease situation in an area, three parallel assays must be run to detect infection due to the *Duttonella, Nannomonas* and *Trypanozoon* parasites. There are many situations which only require knowledge of the presence or absence of trypanosomiasis. To cater for the latter situation and reduce the cost of running the assay, it is desirable to produce a trypanosome genus-specific MAb. Existing anti-trypanosome MAbs were screened for their ability to trap antigens from trypanosome lysates. A few MAbs were found to react with *T. brucei, T. vivax* and *T. congolense* lysates. However, none of the MAbs were able to trap antigens of all three trypanosome species in the sera of infected cattle. Four more MAbs were generated. Two had the capacity to trap trypanosome antigens in sera obtained from cattle infected with three different trypanosome species. The two candidate pan-trypanosome MAbs reveal trypanosome infection

earlier than the species-specific antibodies, and the target antigens seem to persist for a longer period in serum when compared to the half-life of the species-specific antigens. The two candidate antigens will be screened for their reactivity with *Theileria, Babesia, Anaplasma* and *Leishmania* lysates and sera from infected animals.

Currently, the trypanosome species-specific diagnostic MAbs are employed in an antigen-detection ELISA. The assay is of high sensitivity and specificity and applicable in the field. In spite of the ease with which the assay can be employed, production of a cheaper assay, requiring only one reagent, has been investigated. A bispecific antibody, reacting with the host red blood cells as well as trypanosome antigen, would indicate antigenaemia by its agglutination of the host RBC. Mice were immunized with RBC from Boran cattle. The MAbs obtained did not recognize all RBCs. Thus, Balb/C mice were subsequently immunized with RBC from one steer, followed by several booster inoculations with RBC from other cattle. Using this protocol, MAbs were produced, two of which were found to react with the RBCs from 42 Boran cattle so far tested. The two MAbs will be subjected to further scrutiny to determine the polymorphic nature of the epitopes identified by them in other breeds of cattle. It is hoped to combine the pan-reactive anti-bovine RBC MAb with a pan-trypanosome antibody for the development of agglutination assays.

T1b.4 The comparative advantage of antigen-ELISA over routinely used parasitological techniques in the diagnosisof *Trypanosoma brucei* infection and the significance of antigenaemia

Scientists: R. Masake, S. Moloo
Research Associate: S. Minja
Technicians: J. Makau, J. Njuguna

Use of the antigen detection ELISA in the field has revealed the presence of trypanosome antigens in many aparasitaemic animals. It is not known if tsetse can pick up infection from aparasitaemic but antigenaemic animals. Four cattle were therefore inoculated with *T. b. brucei* and monitored for parasitaemia. Trypanosomes were first seen in the blood 17 days after infection. Despite the long prepatent period, all the animals exhibited a first peak of parasitaemia followed by the irregular appearance of parasites in the peripheral blood. These parasitaemias were marked by long aparasitaemic intervals with one of the animals being aparasitaemic for a period of over one year. Sera from the infected animals were examined for the presence of trypanosome antigens, and all the animals were shown to be antigenaemic throughout the course of disease.

One year after infection, four groups of teneral *G. m. morsitans* were fed on the four chronically infected cattle. Thereafter the flies were maintained on rabbits, and on day 30 the surviving tsetse flies were dissected and their infection rates determined. Infection rates of the four groups were 25.5, 23.9, 3.4 and 16.4%. In one group, the maturation rate was 11.9%; while the infection did not mature in the other three groups.

Mice were also subinoculated with blood from the four cattle and examined for the development of trypanosomes. Infection was confirmed in one out of the four cattle. This work has clearly demonstrated the ability of tsetse-flies to acquire trypanosome infection from aparasitaemic but antigenaemic animals. Hitherto, animal inoculation has been considered as one of the most sensitive procedures in the detection of trypanosomes in chronic infections. The failure to detect infection in three out of four animals following subinoculation of blood will be further investigated. This work will be extended to aparasitaemic animals infected with *T. congolense* and *T. vivax* so as to establish their potential as reservoirs of infection for *Glossina* even when apparently aparasitaemic.

T1b.5 Development of monoclonal antibody-based assays for identification of trypanosomes in the tsetse fly (*Glossina* species)

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The standard method for identification and differentiation of trypanosome infections in tsetse is by dissection. This method, however, presents several problems especially that of differential diagnosis of trypanosome species and subspecies in the fly. Monoclonal antibodies (MAbs) have therefore been raised for identification of trypanosome species in *Glossina*.

Several MAbs have been produced against trypanosome insect stages (procyclics and epimastigotes) and utilized in a dot-ELISA capable of differentiating trypanosome vector stages of *T. brucei, T. vivax, T. congolense* and *T. simiae* propagated *in vitro*. A modification of the dot-ELISA has been successfully applied to detect trypanosome infections in the midgut, salivary glands and mouthparts of experimentally-infected *Glossina morsitans centralis* and *G. pallidipes*. Evaluation of the sensitivity of the dot-ELISA assay in terms of the minimum number of trypanosomes that can be detected using procyclics derived *in vitro* indicated that the *T. brucei, T. congolense* and *Nannomonas*-specific MAbs could detect as low as ten organisms per dot, while the *T. vivax* MAbs detected a minimum of 1,000 organisms per dot. The dot-ELISA allowed detection of 90% of *T. brucei*, 95% of *T. congolense* and 94.3% of *T. simiae* infections in experimentally infected *Glossina* midguts, and 93.3% of *T. brucei*-infected salivary glands. The MAbs have been shown not to cross-react with *T. grayi* which also infects the midgut of tsetse. In a preliminary field trial at Galana Ranch, the dot-ELISA test proved to be applicable in the field. Of 52 *G. pallidipes* flies dissected and examined by microscopy, only two were infected, both in the midgut. The infecting trypanosomes were identified as *T. congolense* by dot-ELISA.

T1b.6 Development of non-radioactive and sensitive DNA probes for the detection of trypanosome infections in tsetse flies and livestock

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Simple non-radioactive protocols have been adapted for the detection of African trypanosomes present in the blood of mammalian hosts and the saliva of live tsetse vectors. Commercial kits for the non-radioactive labelling of DNA, with subsequent detection of the labelled probe hybridized to its cognate sequences, were evaluated for their suitability to reveal trypanosomes present in crude samples prepared from infected tsetse and livestock. Of the kits tested, that using a digoxigenin/LumiPhos combination proved to be best in its ability to specifically detect parasites in crude test materials from both infected tsetse flies (midguts and proboscides) and mice. In dot blot assays, a digoxigenin-labelled savannah-type *T. congolense* DNA probe detected an equivalent of ten trypanosomes in a spot on a filter paper after a 15 min exposure of the paper to X-ray film without an intensifying screen at room temperature. It required a one week exposure of the filter to X-ray film with an intensifying screen at –70 °C to obtain similar results when the probe was labelled with [α^{32}P]-dCTP. Once labelled with digoxigenin-11-dUTP, the probe is stable enough to be used repeatedly for a period of one year or longer.

The detection of parasite DNA relies on hybridization with parasite type-specific DNA probes labelled with digoxigenin, followed by amplification and development of the hybridized probe using anti-digoxigenin antibodies conjugated to alkaline phosphatase and the addition of LumiPhos which, when dephosphorylated by the enzyme, results in light emission, and this can be detected by exposure to X-ray film. When used in combination with the polymerase chain reaction (PCR), the methods are sensitive enough to detect trypanosomes present in the buffy coat from antigenaemic, but aparasitaemic cattle and the saliva of live tsetse. Protocols that utilize locally available reagents are currently being investigated, as are methods for linking this assay system with diagnostic PCR.

T1b.7 Molecular characterization of a new genotype of *Trypanosoma (Nannomonas) congolense* from Tsavo, Kenya

Scientist: P. Majiwa
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Trypanosoma (Nannomonas) congolense comprises morphologically identical but genetically heterogeneous parasites infective to livestock and other mammalian hosts; three different genotypes of this parasite have been described previously. In the course of using DNA probes to identify types and species of trypanosomes present in heavily infected cattle and tsetse flies in different parts of Africa, *Nannomonas*-type trypanosomes have been observed that do not hybridize with any of the existing probes. Trypanosomes present in one such infection in a *Glossina pallidipes* caught at the Ngulia Rhino Sanctuary, Tsavo West National Park, Kenya, were isolated successfully in culture directly from the tsetse fly's proboscis. Using this isolate, restriction enzyme fragment length polymorphism (RFLP) analyses of conserved nuclear and kinetoplast DNA sequences together with analysis of randomly amplified polymorphic DNA (RAPD) patterns have demonstrated that the trypanosome is significantly different from the other *T. (N.) congolense* characterized so far at the molecular level. We have identified a highly repetitive, tandemly arranged, satellite DNA sequence from this trypanosome and have shown that a recombinant plasmid containing this DNA can be used as a sensitive probe for the specific identification of the trypanosome. The application of the polymerase chain reaction (PCR), using oligonucleotide primers based on the sequence of the repetitive DNA, makes the probe highly sensitive.

T1b.8 Inter- and intra-species differentiation of trypanosomes through genomic fingerprinting with arbitrary primers

Senior Research Fellow: J. Waitumbi
Scientist: N. Murphy

Selected single short oligonucleotide primers of arbitrary sequence were used to amplify trypanosome genomic sequences using the polymerase chain reaction (PCR). The reaction generated sets of amplified DNA fragments which, when separated by gel electrophoresis, showed polymorphisms for different parasite isolates. Materials that can be used for this method include purified DNA, crude trypanosome lysates and parasites embedded in agarose blocks prepared for pulsed-field gel electrophoresis. The technique could readily differentiate species of morphologically indistinguishable trypanosomes belonging to the *Nannomonas* subgenus, which includes *Trypanosoma simiae*, and four different genotypic

groups of *T. congolense* namely, Kilifi, West Africa forest and riverine, savannah and Tsavo. Among the *Trypanozoon* subgenus, which includes *T. evansi, T. brucei brucei, T. b. rhodesiense* and *T. b. gambiense*, differences in fingerprints between different isolates and subspecies could also be observed. However, only *T. b. gambiense* and *T. evansi* could be unequivocally distinguished from the others. Although distinct differences in karyotype patterns were observed in different *T. evansi* isolates, genomic fingerprinting with arbitrary primers did not generate qualitative polymorphisms in the same parasites. Finally, total PCR products produced in this study could be easily cloned in *E. coli* plasmid vectors. Following transfection of *E. coli* with the plasmid libraries, individual colonies were rapidly screened for plasmids containing inserts, without having to purify them, by performing the PCR using the same primer that had been used to generate the trypanosome PCR products.

In a parallel study, 13 *T. evansi*-like stocks kept at ILRAD were designated as *T. evansi* according to the criterion of homogeneity of kDNA minicircles. The *T. evansi* randomly amplified fingerprints obtained using oligonucleotide primer ILo 525 had a characteristic 283 bp band, which other *Trypanozoon* isolates examined did not have. This band has been sequenced and specific primers could be designed for use in future epidemiological studies for direct identification of *T. evansi* in mammalian hosts and vectors by PCR amplification.

T1b.9 Diagnosis of trypanosome infections in cattle infected with savannah type *Trypanosoma congolense* using PCR

Senior Research Fellow: J. Waitumbi
Scientists: R. Masake, P. Majiwa

Amplification of savannah-type *Trypanosoma congolense* satellite DNA by the polymerase chain reaction (PCR) was used in detection of the parasite in whole blood after erythrolysis with NP40 followed by SDS and proteinase K treatment. In a control experiment, 50 fg (corresponding to about 1/2 a trypanosome) of parasite DNA was amplified in the presence of 10 μg of bovine DNA, and produced a visible band of the expected size upon electrophoresis in agarose gels and staining with ethidium bromide. Higher amounts of host DNA inhibited the specific amplification of parasite DNA. In cattle challenged with tsetse infected with savannah-type *T. congolense*, the presence of the parasite DNA could be detected four days after the fly bite. In comparison, the buffy coat technique did not reveal the presence of these infections until day 12. Further work is in progress to assess the possibility of demonstrating the presence of parasite DNA in cattle infected with Kilifi-type *T. congolense* and *T. b. brucei* and in cattle aparasitaemic but antigenaemic by the antigen-ELISA technique.

T1b.10 Identification of *Trypanosoma vivax* isolates by DNA fingerprinting

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Trypanosoma vivax is an economically important parasite causing diseases in several species of ruminants not only in Africa but also in Central and South America where it has adapted to mechanical transmission by biting flies other than tsetse. Individual isolates of this heterogeneous group are known to differ in characteristics such as pathogenicity, infectivity for laboratory rodents, requirements for cultivation *in vitro*, isoenzyme and karyotypic patterns and reactivity with repetitive DNA sequence probes.

In the quest for simple and direct diagnostic methods for the identification and characterization of *T. vivax* parasites, the polymerase chain reaction method using arbitrarily selected oligonucleotides (AP-PCR) has been employed. Thirty-five primers (of 10 or 11 bases) having GC contents of between 60% and 90% were used. Three of the primers ILo 101, ILo 525 and ILo 1060 gave polymorphic and reproducible DNA fingerprints for all 13 *T. vivax* isolates tested. On the basis of the polymorphisms of the PCR products generated by all three primers, the *T. vivax* could be separated into a Kenyan group and all other *T. vivax* isolates tested (isolated from Uganda, Nigeria, Gambia and Colombia). ILo 525 was the only primer, however, that gave isolate-specific fingerprints. Among the *T. vivax* parasites from Kenya (Bamburi, Galana and Kilifi), a prominent doublet of approximately 870 bp and a single band of approximately 1,100 bp were common to the fingerprints of this group, although minor polymorphisms were also evident between isolates. Two parasites from Uganda (Lugala and Teso) had DNA fingerprints generally similar to those of the parasites from West Africa and South America. Although the *T. vivax* parasites from West Africa had mainly similar DNA fingerprints, the two Nigerian parasites could be distinguished by a 1,400 bp band for ILDat 1.2 (Zaria) which was absent from IL 3185 (Ibadan). The DNA fingerprints of an isolate from Buruku, The Gambia, were more similar to those of the two Colombian parasites obtained from the Andean valleys (Dorada and Palmira) than the rest of the West African parasites. Of the four *T. vivax* isolates from Colombia, the two parasites from the Atlantic coastal plain (Lorica and Monteria) possessed major PCR products of 600 bp and below, and could be differentiated from the pair of isolates from the Andean valleys. Thus, AP-PCR, which is a relatively rapid and sensitive method and requires small amounts of DNA, can be used for the identification and discrimination of *T. vivax* parasites.

T1b.11 Epidemiology and diagnosis of *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei* infections in horses on the Del Monte Farm in Thika

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The Del Monte Kenya Ltd. Co., Thika, Kenya, has 35 horses that are mainly used for security patrols on the pineapple plantations. All these horses were examined at least once a month for a period of 14 months to establish the trypanosomiasis prevalence, incidence and to identify the species of trypanosomes involved. A total of 19 horses showed evidence of disease during this period, 14 of these with clinical symptoms. These included poor body condition, lethargy, fever, an elevated pulse and heart rate, and pale or icteric mucous membranes. Some horses also developed oedema of the dependent parts of the body, particularly the hind limbs. The parasitological tests used in diagnosis detected only *T. congolense* parasites in nine horses, some of which had recurrent infections. All 19 horses were positive for either *T. brucei, T. congolense* or *T. vivax* antigens using the antigen ELISA. In some cases, mixed infections with two or even three of these parasites were detected using this technique. All the infected horses were subsequently treated using diminazene aceturate (Veriben®) at a dose range of 3.5–7 mg/kg, and their response monitored. The animals recovered though, in some cases, a recurrent infection was detected two or more months later with the same or a different parasite.

Characterization of the trypanosomes involved in the outbreak was on the basis of the clinical progression of the disease in experimentally infected horses, morphology and the use of DNA hybridization probes. The parasites were all identified as savannah-type *T. congolense*. Molecular karyotyping may help determine whether the trypanosomes belong to one or more serodemes.

The use of odour-baited traps revealed the presence of numerous *Glossina pallidipes* on the farm as well as a variety of biting flies, including Stomoxys and Tabanid species. Some of the tsetse flies were dissected but no parasites were detected within them. The number of flies caught and the incidence of disease increased in the rainy seasons.

T1c.1 Recombinant loci in natural populations of savannah-type *Trypanosoma congolense*

Scientist: P. Majiwa
Technician: M. Maina

Primers of arbitrary nucleotide sequence have been used to perform polymerase chain reaction (PCR) amplification of DNA from trypanosome clones representing the four different genotypes of *Trypanosoma (Nannomonas) congolense:* the Kilifi-type, the savannah-type, the West African riverine/forest-type and the Tsavo-type. None of the primers used produced randomly amplified polymorphic DNA (RAPD) bands that are identical in both size and quantity among the four genotypic groups of *T. congolense.* This is in accord with the data previously obtained from comparative analyses of the kinetoplast and the highly repetitive DNA sequences. Thus, for comparison of different genetic loci, each genotypic group was treated separately.

Oligonucleotide primers which produced relatively simple patterns of RAPD bands were used in the amplification of DNA from cloned isolates of *T. congolense* representing three distinct and well characterized antigenic repertoires. Several polymorphisms were apparent which included those that were antigenic repertoire- (serodeme-) specific and others that were trypanosome clone-specific. In general, all the clones derived from an antigenic repertoire had an identical pattern of RAPD products. Among such clones, the main differences observed were in the relative amounts of product in a particular band. The pattern of RAPD bands appeared to have no relationship with the chromosome rearrangements apparent among some of the clones within an antigenic repertoire.

Such analyses allow the simultaneous examination and comparison of different loci and can reveal polymorphisms that occur naturally within populations of organisms. Thus, they can be used to detect somatic or sexual hybrids that may have resulted from natural recombination. The random primers were used to search for recombinant loci among the different isolates of the savannah-type of *T. congolense.* A small proportion of the primers used showed what appeared to be recombinant loci. In order to confirm the recombinant nature of the loci and to identify phenotypes, and possibly genes associated with them, some of the relevant polymorphic RAPD bands have been cloned for more detailed analyses. It will be possible also to use such cloned fragments of DNA in determining the extent of polymorphisms at the parasite population level. Similar analyses will be extended to the Kilifi-type *T. congolense* cloned stocks that represent isolates from a closed ecological niche.

T1c.2 Can genetic recombination occur between clones of the Kilifi-type and the savannah-types of *Trypanosoma congolense?*

Scientists: R. Masake, S. Moloo, P. Majiwa
Technicians: A. Adema, H. Gathuo, J. Makau

Genetic exchange occurs in *T. brucei.* Since this phenomenon may have far reaching effects on the characteristics of pathogenic trypanosome populations, a study was conducted to determine whether genetic exchange also occurs between *T. congolense*

parasites. This led to the co-transmission through *Glossina morsitans* of two, well characterized *T. congolense* clones. Trypanosomes resulting from the infection were analysed by DNA hybridization with trypanosome type-specific probes, to identify populations consisting of a mixture of the two clones. Trypanosome clones were prepared from parasite populations which hybridized with both of the DNA probes, each specific for either of the two parental clones in the mixture used to infect tsetse. The clones were hybridized with a DNA probe specific for either savannah-type or Kilifi-type *T. congolense*. All the progeny clones examined so far hybridize with only the savannah-type *T. congolense* DNA probe. Twenty-five of the clones were screened for variability in chromosome sizes by orthogonal field alternating gel electrophoresis. All the clones displayed chromosome patterns similar to those of savannah-type *T. congolense* clone IL 1180, except for minor variations in the medium-sized chromosomes seen in five of the clones. Thereafter, the isoenzyme patterns of IL 1180, K45/1 (Kilifi-type) and the five clones were compared. Four out of five had isoenzyme migration patterns identical to that of IL 1180. One differed from both IL 1180 and K 45/1 in the mobility of peptidase 1 (PEP 1). The same clone also gave rise to different size products following polymerase chain reaction (PCR) using an arbitrary oligonucleotide primer ILo 509. This clone will be further investigated to ascertain whether or not the differences observed are consistent and reproducible.

In addition to the co-transmission experiment, 110 oligonucleotide primers were examined in an effort to identify those that might allow the distinction of clones of the Kilifi-type *T. congolense* belonging to different serodemes. The majority of the primers produced similar PCR products for two clones (K45/1 and K60/1) while K97/2 had a few more products which differed in size from the other two clones. Nevertheless, there were five primers which gave products of different size and number of DNA products for each of the three clones. These primers were employed in examining seven other Kilifi-type *T. congolense* clones. However, none of them was useful in differentiating the clones. More of the Kilifi-type *T. congolense* clones are being screened by arbitrarily primed PCR.

T1c.3 Comparative analysis of loci in *Trypanosoma congolense* clones that differ in tsetse transmissibility

Scientists: P. Majiwa, S. Moloo

Biochemical and immunological studies of many processes in parasitic protozoa routinely require the use of a large number of organisms which are homogeneous in the properties under study. In a majority of cases, the African trypanosomes recently isolated from infected hosts or vectors do not readily grow to high parasitaemia in the commonly used laboratory rodents. An approach normally taken to obtain large numbers of such parasites involves performing several rapid syringe passages in laboratory rodents until a high parasitaemia is achieved in the rodents. During the manipulations in the laboratory, the parasite will often undergo phenotypic changes which may include an alteration in the rate of antigenic switching, impaired capacity to generate pleomorphisms and inability to complete cyclical development in the tsetse vector. It is important to understand the genetic basis for these phenotypic changes.

Genomes of parasitic protozoa contain various repetitive DNA sequence elements organized either in long arrays of tandem repeats or in a small number of units dispersed throughout the genome. In trypanosomes, repetitive DNA sequences play a central role in genome rearrangements that facilitate adjustments necessary for survival in different environments. The best characterized sequence-mediated gene rearrangement in the African trypanosomes is that associated with the expression of the variable surface glycoproteins (VSGs) on the surface of the trypanosomes. The rearrangements appear to be necessary for switching to the expression of an alternate VSG during antigenic variation.

Using randomly amplified polymorphic DNA (RAPD) analysis and Southern blot hybridizations with a panel of recombinant DNA probes, several discrete loci in trypanosome clones derived from a single primary isolate but separated by ten years have been compared. One of the cDNA clones identified a locus which appears to be either deleted or grossly rearranged in the trypanosome clone which had been passaged many times. This trypanosome is unable, or has an extremely diminished capacity, to complete cyclical development in the tsetse vector. The main features of the locus and its dispersion among the different cloned isolates of savannah-type *T. congolense* are being investigated.

T1c.4 Clonal growth of *Trypanosoma brucei* GUTat3.1 and ILTat1.4 and *Trypanosoma congolense* IL1180 and IL 3000 on agarose plates

Scientists: V. Carruthers*, H. Hirumi, P. Majiwa, N. Murphy
Research Associate: K. Hirumi
Technicians: J. Wando

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Recently, Carruthers and Cross (in press) developed a technique for the clonal growth *in vitro* of both bloodstream and procyclic forms of three clones of *Trypanosoma brucei*, derived from Stock 427, on agarose plates using HMI-9 medium (developed for cultivating *T. brucei* bloodstream forms in an axenic culture system) and SDM-79 medium (used for cultivating procyclic forms), respectively. This technique opens the way for genetic analyses of African trypanosomes in a manner similar to that employed for yeast. The applicability of the technique to bloodstream and procyclic forms of *T. brucei* GUTat3.1 and ILTat1.4 and *T. congolense* IL1180 and IL 3000 was examined.

The results demonstrated that whilst the technique is applicable to the clonal growth of the *T. brucei* bloodstream forms, it is inappropriate for the growth of the *T. brucei* procyclic forms, as well as of the *T. congolense* bloodstream forms. Thus a modification of the method was made by replacing HMI-9 medium with HMI-93 medium and SDM-79 medium with TBM-5 medium. The agarose plates modified with HMI-93 medium successfully supported the clonal growth of the *T. congolense* bloodstream forms, although further improvements were needed for the procyclic forms. The method for *T. congolense* bloodstream forms will be used in transfection experiments when plasmid constructs become available.

T1d.1 The use of arbitrary primers for the identification of developmentally regulated genes

Scientists: N. Murphy, R. Pelle
Technicians: P. Pandit, S. Wasike

It is estimated that of about 100,000 potentially different genes encoded in the higher eukaryotic genome, only a small fraction, about 10,000, are expressed in any individual cell. All life processes, such as cell division, differentiation and development, are driven by changes in gene expression. Current techniques for the identification of developmentally regulated genes employ various methods for the substractive enrichment of cDNA, generated from mRNA, which is specific to one life cycle or cell-cycle stage. These techniques require relatively large quantities of material, are labour intensive and are restricted to the identification of developmentally regulated genes that are expressed at a high level. Many of the most important genes involved in cell-cycle regulation, proliferation and differentiation do not generate abundant transcripts. There is therefore a requirement for the development of improved systems for the identification of developmentally regulated genes.

Short oligonucleotide primers (10-mers) of arbitrary sequence are currently used in the polymerase chain reaction (PCR) to generate genomic fingerprints for the characterization and differentiation of organisms and for mapping loci encoding genes which confer identifiable phenotypes. It was hypothesized that the use of such primers on cDNA may generate fingerprints which are characteristic of the genes being expressed. To test this hypothesis, *T. b. brucei* was used because of the clear differentiation of atively dividing bloodstream-form trypanosomes from non-dividing forms in this species. In this technique, mRNA from each form is first reverse transcribed into cDNA and this is used as the template for arbitrary primer amplification. A small amount of cDNA (10–20 ng) is mixed with a single 10-mer primer and the PCR is carried out for 30 to 40 cycles. The fingerprints of DNA fragments generated are then examined by agarose gel electrophoresis. The technique generates reproducible fingerprints with different primers, and some primers generate numerous polymorphisms for the different forms of the parasite used. That the presence of an amplified DNA fragment in one form and not another is due to specific expression in this form has been shown through Northern blot analysis with labelled fragments. Using this technique, a gene has been identified which is only transiently expressed as *T. b. brucei* transforms from an actively-dividing form to a non-dividing form.

In a variation on this technique, arbitrary primers have been used together with a specific primer for the miniexon or spliced-leader sequence found at the 5′ end of all trypanosome mRNAs. Again the PCR products are analysed by agarose gel electrophoresis and DNA fragments showing differences between stages are purified and used as probes on Northern blots. Using this approach, amplified cDNA fragments have been identified which hybridize specifically to slender bloodstream form transcripts, stumpy bloodstream form transcripts and procyclic form transcripts. These results demonstrate the power of the arbitrary primer amplification of cDNA PCR (APAC-PCR) technique for the rapid identification of developmentally regulated genes. Further modifications to the technique, such as the use of

pairs of arbitrary primers or oligo d(T) with an arbitrary primer etc., will increase the number of possibilities for identifying differentially controlled genes in trypanosomes.

T1d.2 Studies of *cdc* genes of *Trypanosoma congolense* and the use of arbitrary primers to identify differentially expressed genes during the cell division cycle

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Research Associate: K. Hirumi
Technicians: P. Pandit, A. Muthiani, F. Chuma
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In earlier studies, potential homologues for several different classes of *cdc* genes have been identified and their DNA sequences have been partially or completely deduced, in some cases for several different species. For *T. congolense* (savannah), two potential *cdc*2 homologues have been identified, one of which (*Tckin*1) has been focused on due to it greater similarity to *cdc*2 from other species. This homologue has also been identified in *T. congolense* (Kilifi), *T. vivax, Leishmania mexicana* and *T. brucei*. 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. Genes related with the PCTAIREIS box have now been identified in humans and it has been suggested that, since similar genes have not been reported for lower eukaryotes such as yeasts, the different *cdc*2-like genes are required and differentially modulated in the different cell types of a multicellular organism. Results from the Glasgow group and from experiments at ILRAD lead us to conclude that there are at least two *cdc*2-like protein activities in trypanosomes that have differential activities at different life-cycle stages.

Studies on the transcription of *Tckin*1 show that at least two closely linked upstream genes also generate transcripts, and could be expressed together with *Tckin*1, as part of one transcription unit. Sequence analysis of this upstream region has to date revealed the presence of a small ribosomal protein, the *T. congolense* homologue of the S12 protein, which is involved in the regulation of translation. This finding potentially associates the *Tckin*1 gene with a gene encoding a product involved in the regulation of translation.

Attempts to complement a *Schizosaccharomyces pombe* temperature sensitive *cdc*2 mutant with both the *L. mexicana* and *T. congolense* genes have been unsuc-

cessful, and experiments with the human homologues of these genes report similarly negative results. The conclusions from these findings have been that yeasts do not have a homologue for these genes and the findings reflect the problems of working in heterologous systems. Attempts to initiate proliferation in starfish oocytes with the *Tckin*1 fusion protein work carried out (at Banyuls sur Mer) have also not been successful.

Recent reports have demonstrated that the previous suggestion that transcription of the human *cdc*2 gene is not regulated are inaccurate. Transcription of this and other cell cycle genes are regulated during the cell division cycle as has now been observed in synchronized populations of cells. These results have encouraged the search for genes that are transcriptionally regulated during the cell division cycle using synchronized *T. congolense* parasites prepared *in vitro*. The degree of synchronization of these cells has been examined microscopically and by fluorescence activated cell sorter. Trypanosomes were harvested at 0, 2, 4, 6, 8 and 12 hours, following initiation of growth for the first batch and at 0, 4, 8, 10, 12, 14 and 16 hours for the second batch. Approximately 10^7 trypanosomes for each time point were used for total RNA isolation and half of this was used to generate cDNA. Since the cDNA generated from such a small number of cells is not sufficient to carry out any form of analysis on differential gene expression, we exploited the principal and unusual feature of trypanosome mRNAs in that they contain fixed 5′ and 3′ ends and amplified half of the generated cDNA in the polymerase chain reaction (PCR) with oligo d(T) and miniexon primers. The resultant cDNA (approximately 10 µg for each time point) is sufficient to carry out several thousand PCRs with arbitrary primers [see abstract T1d.1]. Initial results suggest that the technique can rapidly identify cell cycle regulated genes of interest.

T1d.3 The identification of genes in *Trypanosoma brucei brucei* involved in the establishment of infection in a mammalian host

Scientists: N. Murphy, H. Hirumi
Research Associate: K. Hirumi
Technician: P. Pandit

Previous reports have described a number of attenuated *T. brucei* GUTat 3.1 mutants which have a normal doubling time *in vitro*, but are unable to establish an infection in an immunocompetent mammalian host. These mutants represent ideal material for testing systems for the generation of revertants since there is a direct selection system for revertants (i.e. growth in a mammalian host). The generation of mutants with easily identifiable phenotypes is an important tool in genetic analysis. However, the ability to generate revertants and map the genomic location at which an alteration has occurred which correlates with the change in phenotype is considerably more powerful. Transfection of total DNA, with an average size of 30 kbp, from a virulent ILTat 1.1 parasite resulted in three independent revertants whose chromosome profile appeared identical to the parental attenuated population. In order to identify the genomic location where the alteration occurred, we utilized arbitrary primers in the polymerase chain reaction (PCR) on genomic DNA

from the virulent parental, the avirulent mutant and the three revertant transfectants. One of the primers amplified a fragment in the virulent parental and the three transfectants but not in the avirulent mutant. This PCR product has been cloned and sequenced at both ends. One end of the fragment contains sequences of the TRS1/ingi transposable element, but the sequence identity of the other end has not yet been established.

T1d.4 Identification of regulatory proteins involved in post-transcriptional control in trypanosomes

Scientists: R. Pelle, N. Murphy
Collaborating Scientists: E. Pays*, P. Tebabi*
Technician: S. Wasike

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Recent reports suggest that gene expression in African trypanosomes is mainly controlled at the level of RNA processing. One of the most important types of RNA processing in trypanosomes is the trans-splicing of nuclear mRNAs by which the miniexon from medRNA is ligated to the 5′ end of pre-mRNAs. This process involves interactions between small nuclear RNAs and cytoplasmic proteins, transport of RNA-protein complexes from the cytoplasm to the nucleus, and formation of a spliceosome with the pre-mRNA. Proteins involved in this splicing event in particular, and specific RNA binding proteins in general, may play an important role in the regulation of gene expression and cell proliferation. Thus, the identification of trypanosome RNA binding proteins may lead to a better understanding of the post-transcriptional control of gene expression. Because the number of different proteins interacting with RNA is fairly large, it becomes important to identify which of these proteins make direct contact with the RNA, as some proteins may be associated with RNA via protein-protein interactions.

For this purpose a UV-cross-linking hybridization technique has been developed that allows easy detection of complexes formed by RNA and proteins in close contact *in vivo*. In the development of this technique, model genes which generate abundant transcripts have been used. The results clearly demonstrate that *T. b. brucei* medRNA and VSG mRNAs are associated with cytoplasmic proteins. Some of the complexes that are formed by medRNA and its binding proteins are present at a high level in the procyclic form, but only at trace or undetectable levels in bloodstream forms. The results suggest that proteins involved in binding specifically to medRNA are likely to be differentially regulated themselves in the different life-cycle stages. This possibility can now be tested using this approach.

In collaboration with Dr. E. Pays, it has been found that the 300 nt-3′ end of the AnTat 1.10 VSG mRNA forms specific complexes with cytoplasmic proteins from both GUTat 3.1 and ILTat 1.1 bloodstream forms. This complex is not formed when using cell extracts from procyclic forms of the same clones. However, the complex is also formed, although not as abundantly, when antisense RNA to the 300 nt-3′ end VSG mRNA is used. Although this was a surprising finding, results on mRNA

stability and expression of a reporter gene in transient transfection experiments conducted in Brussels support these findings. The mRNA sequence involved in binding these proteins is suspected to involve the conserved 14 nt found at the 3′ end of all *T. b. brucei* bloodstream form VSGs. Removal of this sequence has been found to decrease mRNA stability together with transient expression of a reporter gene in Brussels. On the basis of our previous results, it is of interest to know whether a decrease in the binding of cytoplasmic proteins is associated with the removal of the 14 nt sequence. Thus, the VSG mRNA complex will be used to raise antibodies against VSG mRNA binding proteins.

T1d.5 Cytoskeletal genes and encoded proteins of *Trypanosoma congolense*

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The genes encoding cytoskeletal (CSK) proteins of *T. congolense* Tcp75G1 and Tcp75G2 have been characterized. These CSK proteins, when analysed by SDS-PAGE, run as a doublet and are encoded by a multigene family. The Tcp75G1 and Tcp75G2 display 65% identity at the amino acid level and are localized to the paraflagellar rod. The gene products have multiple potential sites for glycosylation, phosphorylation by a variety of protein kinases and myristilation. However, Tcp75G2 is a truncated (5′) version of Tcp75G1, a process which might have arisen from incomplete gene duplication. The two genes would appear to have subsequently undergone divergent evolution.

The third member of the *T. congolense* CSK protein family that has been studied is Tcp53. Antibodies selected by clones expressing this gene have been used to localize the product by immunoelectron microscopy. Fusion proteins with β-galactosidase (pUR 291) have been produced and antifusion protein antibodies have also been raised in rabbits. These antibodies recognize Tcp53 on Western blots and have localized this protein predominantly to the region between the basal bodies, the kinetoplast and the flagellar pocket. There is, however, some antibody reactivity with the paraflagellar rod, suggesting a degree of cross-reactivity with Tcp75G1/2.

The cDNA encoding this protein has been partially sequenced and reveals a predicted protein sequence containing domains with 68% identity to calmodulins from *T. cruzi* and *T. b. gambiense*. The calcium-binding sequence motif DKDGDGTITTKE, which is present in the calcium-binding domain I, is thus completely conserved among all the three trypanosome species. Sequence comparisons of Tcp53 with proteins from the database indicate that it may, like parvalbumin with which it shares a significant degree of identity, be a multi-functional protein since it also has domains that exhibit homology with transport proteins, Ca^{2+}ATPases and with the middle T antigen of mouse polyoma virus.

T1d.6 Characterization of an expression site for a metacyclic form stage-specific variable antigen gene of *Trypanosoma (Nannomonas) congolense*

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A region in the immediate upstream vicinity of a metacyclic-form stage-specific variable surface glycoprotein (mVSG) gene of *T. congolense* IL 3000 has been analysed. Restriction enzyme digestion and Southern blot hybridization analysis have revealed that the expression site of this gene does not contain significant stretches of DNA sequence devoid of restriction enzyme sites, nor does it contain highly repetitive DNA sequences analogous to those observed in the vicinities of some of the VSG genes of *T. brucei*. Northern blot hybridizations have indicated the presence of at least two distinct transcripts, each from a different segment of the region upstream of the mVSG gene. Both of the transcripts have been obtained as clones from a λgt11 cDNA library of IL 3000 metacyclic forms. Transcription of each of the mRNAs is developmentally regulated, being active only in the metacyclic forms of the parasite. Preliminary sequence analysis of the transcript nearest to the mVSG1 gene indicates that it encodes a protein highly homologous with DNA-binding proteins and adenylate cyclase.

Unlike sequences which encode a majority of the different *T. congolense* VSGs analysed to date, the mVSG1 gene and its upstream flanking sequences are conserved in at least one other antigenic repertoire, ILNaR 3. This is the first evidence at the molecular level that indicates the possible existence of isotypic antigenic types (iso-VATs) among trypanosomes within the *Nannomonas* subgenus.

T1d.7 Synchronization of the cell division cycle of *Trypanosoma congolense*

Scientists: H. Hirumi, N. Murphy
Research Associate: K. Hirumi
Technicians: A. Muthiani, J. Wando

Although earlier attempts to synchronize the cell division cycle of *T. brucei* and *T. congolense* by chemical treatments using hydroxyurea, theophylline and D-sorbitol were unsuccessful, partial synchronization of *T. congolense* IL 3000 has been achieved by regulating conditions for cultivating metacyclic and bloodstream forms in a combination of two axenic culture systems *in vitro*.

To date, the best synchronization from the non-dividing stage (metacyclic stage) to the dividing stage (bloodstream form) was achieved by (1) producing metacyclic forms in epimastigote cultures in an axenic culture system at 27 °C using TBM-5 medium, (2) isolating metacyclic populations from 45–50-day-old cultures by means of DE-52 column chromatography, (3) maintaining the metacyclic populations for an additional 16 hours under the culture conditions for insect forms, (4) transferring the metacyclic populations to an axenic culture system for bloodstream forms and (5) maintaining the cultures at 34 °C using HMI-93 medium for an additional 24 hours.

The majority (> 99%) of metacyclic forms remained at the non-dividing stage for 16 hours under the insect form culture conditions. These non-dividing metacyclics, however, rapidly transformed to bloodstream forms and underwent the division cycle in a semi-synchronized manner when the insect form culture condition was lifted and the trypanosome populations were maintained under bloodstream form culture conditions. After eight hours under the bloodstream form culture conditions, more than 64% of trypanosomes were at the early stage of cell division (61.5% with two kinetoplasts and a single nucleus, 2.8% with two kinetoplasts and two nuclei). In contrast, 48% of trypanosomes were at the dividing stage (22.4% with two kinetoplasts single nucleus, 25.6% with two kinetoplasts and two nuclei) in a control group which was directly maintained under the bloodstream form culture condition for 24 hours.

T1e.1 Longitudinal study of drug-resistance phenotype and genotype of *Trypanosoma evansi* originating from camels in northern Kenya

Senior Research Fellow: J. Waitumbi
Scientists: A. Peregrine, N. Murphy

Thirty-six isolates of *Trypanosoma brucei*-type trypanosomes were collected from camels in northern Kenya during the dry season in 1986 and during the wet season in 1987, and were designated as *T. evansi* according to the criterion of homogeneity of kinetoplast DNA minicircles. Although all the isolates had indistinguishable minicircles, polymorphism in chromosome-sized DNA molecules, as detected by electrophoresis, was extensive. The isolates could be grouped into eight distinct electrophoretic karyotypes. All isolates, except those that were collected from a camel herd with a long history of trypanocide application and under chemoprophylaxis at the time the isolates were collected, belonged to one karyotypic group. From a second herd in which trypanosomiasis management was by individual treatment of proven parasitaemic cases, isolates with diverse karyotypes were obtained. Some of the karyotypes identified during the dry season in both herds were re-isolated either in the same or different animals in the same herd in the subsequent wet season. The observations indicated that distinguishing *T. evansi* isolates by molecular electrophoretic karyotypes is more sensitive than kDNA analysis. They also indicated that drug pressure selected for certain trypanosome populations.

In other work, 12 isolates of *T. brucei*-type trypanosomes were collected from camels in Samburu, northern Kenya, in 1990–91, after the infections proved refractory to subcutaneous treatment with quinapyramine prosalt at a dose of 5 mg/kg body weight (bw), subcutaneous treatment with cymelarsan at a dose of 0.25 to 0.5 mg/kg bw, and intravenous treatment with isometamidium chloride at a dose of 0.5 mg/kg bw. The 12 isolates were analysed by pulsed-field gel electrophoresis (PFGE), the random amplified polymorphic DNA technique using the polymerase chain reaction (PCR), and on the basis of restriction enzyme digests of kinetoplast DNA minicircles. On the basis of homogeneity of kDNA, all the isolates were designated *T. evansi*. Furthermore, all the isolates had the same electrophoretic karyotype pattern, which in an earlier study had been described as type 'E'. The source of these parasites could be traced to camels that were brought into the herd in 1988 since the herd from which the animals originated had previously been shown to be infected with *T. evansi* of the 'E' karyotype pattern. All the isolates were found to be resistant to quinapyramine sulphate at the maximum dosage tested (10 mg/kg bw) in mice.

Finally, eight *T. evansi* isolates were also collected from a camel herd in Olturot, Marsabit District, in 1990, in which control of trypanosomiasis was by prophylactic use of quinapyramine prosalt. The isolates were predominantly of the 'F' karyotype. In 1986/1987 isolates were collected from the same herd and were also of the 'F' karyotype. It therefore appeared that the predominant karyotype within the herd had remained stable for three years. All isolates were found to express resistance to quinapyramine sulphate at the highest dosage tested (10 mg/kg bw) in mice. Multiple prolonged rat passages (three months) of one of the stocks with the 'F' karyotype pattern, and a clone derived from it, did not alter the karyotype-pattern nor the drug resistance phenotype.

In studies to compare the 'E' and 'F' karyotypic groups, DNA fingerprints generated using the arbitrarily primed polymerase chain reaction did not reveal polymorphisms between the isolates within either the 'E' or 'F' karyotypic groups.

T1e.2 A method for determining the sensitivity of *Trypanosoma congolense* bloodstream forms to trypanocidal drugs *in vitro*

Scientists: H. Hirumi, A. Peregrine
Research Associate: K. Hirumi

The method reported briefly in 1991 for quantifying the sensitivity of bloodstream forms (BSFs) of *T. congolense* to trypanocidal drugs *in vitro* was further improved and standardized. Using the standardized method the sensitivities of BSFs of six stocks and 18 clones of *T. congolense*, propagated in HMI 93 medium in an axenic culture system (Hirumi and Hirumi, 1991), to diminazene aceturate (DA), homidium chloride (HC), isometamidium chloride (IC) and quinapyramine sulphate (QS) were examined in 24-well test plates. Levels of the sensitivity for each drug were expressed in ten steps from ten to one at final concentrations of DA: 600, 500, 400, 300, 200, 100, 80, 60, 40 and 20 μg/ml, and HC, IC and QS: 10-fold serial dilutions from 10 mg to 0.01 pg/ml, all in HMI 93 medium.

Phase-contrast microscopic examination, and colourimetry of the culture media (containing phenol red) (a) confirmed the previous observation that sensitivities of the trypanosome populations to the four drugs can readily be quantified in this system, (b) revealed that the resistance characteristics of each trypanosome population to each drug appeared to occur independently of each other, and (c) demonstrated that the levels of drug sensitivity of each population are stable in culture during 12 months of cultivation. The information obtained in this system using three test plates per drug per trypanosome population for five days provided an equivalent amount of information as testing *in vivo* which uses 36 mice and lasts two months.

T1e.3 Prophylactic effect of isometamidium chloride (Samorin®) in Boran cattle challenged with drug-resistant and drug-susceptible populations of *Trypanosoma congolense*

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Scientists: A. Peregrine, S. Moloo, P. Holmes†
Technician: S. Kemei

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In order to provide information on the relationship between the concentration of isometamidium in cattle serum and susceptibility of animals to challenge, 15 Boran cattle were divided into three groups of five animals each (Groups 1, 2 and 3). On

day 0, all 15 animals were treated intramuscularly with isometamidium chloride (Samorin®) at a dose of 1.0 mg/kg body weight (bw). Additional Boran cattle, kept under identical conditions, functioned as untreated controls. Blood samples for use in the isometamidium ELISA [see abstract T1e.4] and for evaluation of various biochemical parameters (total serum protein, serum albumin and trypanosome antigen) were collected from all animals prior to drug treatment, daily for the first week after treatment, biweekly for a further two weeks and weekly thereafter. Serum was separated and stored in borosilicate glass tubes at –60 °C. At monthly intervals following treatment, cattle were challenged via the bites of *Glossina morsitans centralis* (5/animal) infected with one of three different populations of *T. congolense:* animals in Group 1 with IL 3893 (a stock from Ghibe, Ethiopia); animals in Group 2 with IL 3889 (a stock from Nguruman, Kenya); and animals in Group 3 with IL 1180 (a clone from Tanzania). Whilst IL 3893 and IL 3889 express high levels of resistance to isometamidium *in vitro,* IL 1180 expresses a high level of sensitivity. Following each challenge, cattle were monitored for the presence of trypanosomes using the buffy-coat phase-contrast technique. Cattle found to be parasitaemic were not subsequently challenged with infected tsetse flies but remained on experiment until their packed red blood cell volume (PCV) decreased to below 15%.

Following the one-month challenge, all animals challenged with IL 3893 and all animals challenged with IL 3889 became parasitaemic. While the parasitaemia prepatent period for animals challenged with IL 3893 (Group 1) varied from 16–18 days, animals challenged with IL 3889 (Group 2) were not parasitaemic until 25–35 days following challenge. In contrast, untreated challenge controls for both *T. congolense* populations exhibited shorter prepatent periods. In contrast to animals in groups 1 and 2, animals challenged with IL 1180 (Group 3) were not detected parasitaemic until following the sixth monthly challenge. Thus, whilst isometamidium chloride at a dose of 1.0 mg/kg bw conferred less than one month's prophylaxis to cattle against both IL 3893 and IL 3889, the same dosage conferred complete prophylaxis against IL 1180 for five months. For animals in Group 1, trypanosome antigen was first detected 14 days after infection and fluctuated in titre for the 140 days following infection. For animals in Group 2, trypanosome antigen was first detected on day 21 post-infection and was detected at low levels over the same period. For animals in Group 3, trypanosome antigen was not detected until after the sixth monthly challenge. Finally, in all animals that became infected, infections were characterized by progressive anaemia, hypoproteinaemia and hypoalbuminaemia.

T1e.4 Development of an ELISA for isometamidium

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Collaborating Scientists: P. Stevenson†, S. Maloo‡, W. Thorpe‡

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Development of the isometamidium ELISA has now progressed to the stage of validation of the assay for field use. The assay technique has been refined, with improvements in the preparation and storage of isometamidium-spiked standards in normal bovine serum. These standards may be pre-diluted in buffer containing isometamidium-specific antibody and dispensed onto ELISA plates in the same way as serum samples. The optimum type of ELISA plate (Immulon 4, Dynatech) for the assay has been selected on the basis of assay robustness.

Methods for statistical analysis of the results of the isometamidium ELISA have been developed by adapting techniques used in other areas of immunoassay. Microcomputer spreadsheet software has been developed to perform many of these analyses. In particular, this includes four-parameter logistic calibration curve fitting with calculation of concentrations of unknowns, analysis of variance and precision profiling.

The assay has been used to test serum isometamidium concentrations in experimental cattle following a single intramuscular injection at 1.0 mg/kg body weight and subsequent monthly tsetse challenge with one of three populations of *T. congolense* (IL 3893, IL 3889, IL 1180) of varying degrees of resistance to the drug. Cattle challenged with either IL 3893 or IL 3889 became infected following the first challenge at one month post-treatment. Cattle challenged with IL 1180 remained uninfected following at least five monthly challenges.

A similar pattern of drug elimination to that seen in previous experiments was observed. Isometamidium concentrations were generally similar in all animals until infections were detected. However, in animals which succumbed to infection following the first challenge, the terminal phase half-life was reduced from 22–24 days in uninfected animals to as little as 14 days in animals infected with the most resistant trypanosome population (IL 3893). At the time of the first monthly challenge isometamidium concentrations ranged from 2.7 to 12 μg/ml (mean 6.0 μg/ml). At the time of the third monthly challenge, with IL 1180, which all cattle resisted, isometamidium concentrations ranged from 0.51 to 0.89 μg/ml (mean 0.74 μg/ml). Hence, the difference between the lowest serum concentrations to which IL 1180 was shown to be sensitive and the highest serum concentrations to which IL 3893 and IL 3889 were shown to be resistant was approximately tenfold.

Testing of sera collected from cattle at two different field sites in Kenya, one with natural tsetse challenge and one under carefully managed conditions of isometamidium prophylaxis (Nguruman and Mtwapa), has been carried out. Serum concentra-

tions of isometamidium were found to be similar to those seen in experimental cattle and were generally within the dynamic range of the assay. The assay showed that the reason some animals became infected in spite of prophylaxis was not due to lower circulating drug concentrations, thereby providing indirect evidence for the presence of isometamidium-resistant trypanosomes.

T1e.5 Characterization of anti-isometamidium monoclonal antibodies

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Student: M. Eisler*
Technician: E. Gault*

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In earlier work, murine anti-isometamidium monoclonal antibodies were prepared using an isometamidium (ISMM) human serum albumin-glutaraldehyde conjugate. The antibodies are of the IgG_1 and IgG_{2b} isotypes, trap-free isometamidium and do not cross-react with human serum albumin. In further work, three of the monoclonal antibodies were tested for their suitability for use in the isometamidium ELISA [see abstract T1e.4].

Competition with isometamidium was examined using the above ISMM-human serum albumin glutaraldehyde conjugate to coat ELISA plates. Using this system, approximately similar results were obtained with each of the three monoclonal antibodies. However, monoclonal antibody $SA_3$141.8.6 produced the most sensitive ELISA, with 50% competition occurring at approximately 10 µg/ml. Because the inhibition curve was extremely steep, this was approximately the minimum detectable concentration. Thus, use of any of the three monoclonal antibodies in the ELISA would result in a significant reduction in the assay's sensitivity and the drug concentration range over which it would be able to quantify the drug concentration.

In studies to examine the specificity of the three monoclonal antibodies, competition with isometamidium, homidium and diminazene was examined using the above system. All three trypanocidal drugs competed for all three monoclonal antibodies at approximately the same level. On the basis of the drug concentrations required to produce 50% competition, the cross-reactivity of each of the monoclonal antibodies for diminazene was approximately 0.1%. The cross-reactivity for homidium was slightly less. Since diminazene occurs *in vivo* at concentrations approximately 10^3 times greater than the concentrations of isometamidium, such cross-reactivity may pose problems when samples are collected from animals during the first few days following treatment with diminazene. In order to prevent the occurrence of such false-positive results, an immunological reagent with a maximum level of diminazene cross-reactivity of 0.01% will be required.

T1e.6 Pharmacokinetics of diminazene in cerebrospinal fluid, plasma and lymph of goats

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Earlier work has shown that when *T. congolense* IL 3274 is transmitted to goats via the bites of infected tsetse flies, the population is resistant to a single intramuscular (i.m.) treatment with diminazene aceturate at a dose of 7.0 mg/kg body weight (bw) when administered 19 days after infection (approximately three days after the first detection of trypanosomes). In contrast, when goats were infected in an identical manner and treated with the same drug dosage 24 hours after infection, all animals were cured. There appeared to be a significant alteration in drug sensitivity between day 1 and day 19 of infection. In order to determine whether invasion of the central nervous system (CNS) contributed to this phenomenon, experiments were conducted in goats to determine whether *T. congolense* IL 3274 accesses the cerebrospinal fluid (CSF) and to define the pharmacokinetics of diminazene in CSF. An epidural cannula was placed in the lumbar subarachnoid space of each goat. Seven such goats were infected with *T. congolense* IL 3274 via the bites of infected tsetse flies and treated i.m. three days after first detection of parasitaemia with diminazene aceturate at a dose of 7.0 mg/kg bw. Cerebrospinal fluid samples were obtained from the goats on a daily basis from three days prior to infection to seven days after the trypanosomes had reappeared following treatment. All samples were analysed for *T. congolense* antigen using a species-specific antigen-detection ELISA; all samples were negative for antigen. The data indicated that invasion of the CNS did not contribute to the resistance phenotype. In a second experiment, five uninfected cannulated goats were treated i.m. with diminazene aceturate at a dose of 3.5 mg diminazene base/kg bw. Blood and CSF samples were collected from all treated goats, as well as three cannulated untreated goats, from 0–48 hours following treatment. The CSF samples were analysed for the presence of diminazene by high-performance liquid chromatography (HPLC). Concentrations of diminazene in the CSF were on average three to four times lower than those determined in plasma at the same time point. Peak concentrations (C_{max}) in the CSF averaged 1.04 ± 0.81 µg/ml, as compared to 4.53 ± 0.41 µg/ml in the plasma. The time (T_{max}), 0.44 ± 0.09 hours, at which C_{max} was attained in the plasma was considerably earlier than the T_{max}, 2.0 hours, determined for C_{max} in CSF. Unlike plasma samples, CSF samples could not be aspirated at all the times required for an exhaustive pharmacokinetic study. However, the data indicated that diminazene accesses the CNS, but attains significantly lower levels than in plasma. Thus, if trypanosomes did access the CNS, they would be less likely to be eliminated than if located in the systemic blood circulation.

In a third experiment, the kinetics of diminazene were determined in the lymph of goats in order to determine whether the apparent sensitivity of *T. congolense* IL 3274

on day 1 is due to a significantly higher concentration of diminazene at the level of the skin than that which is attained in the blood. After single i.m. administration of 3.5 mg diminazene base/kg bw, whole blood and lymph samples were obtained at several time intervals from three goats that had been cannulated via the efferent pre-scapular lymphatic duct and had the lymph node removed. In addition, whole blood samples were collected from three non-cannulated goats that received treatment with diminazene aceturate at the same dosage. All samples were analysed for diminazene by HPLC. Analysis of the pharmacokinetic data indicated that the C_{max}, 1.21 ± 0.58 µg/ml, attained in the lymph, was significantly lower than that attained in the plasma of cannulated goats, 3.64 ± 0.61 mg/ml, and in the plasma of non-cannulated goats, 4.53 ± 0.41 µg/ml. Furthermore, the plasma elimination half-life of diminazene in cannulated goats (11.77 hr) was significantly faster than in non-cannulated goats (47.86 hr) and in the lymph (926.11 hr). Consistent with these values, the total body clearance (CL), as determined from the plasma data of cannulated goats (1.76 ± 0.11 ml/min/kg), was significantly faster than in non-cannulated goats (0.69 ± 0.11 ml/min/kg) and in the lymph (0.13 ± 0.11 ml/min/kg). The data therefore indicate that the sensitivity of *T. congolense* IL 3274 to diminazene administered 24 hours after tsetse challenge was not due to higher levels of diminazene at the level of the skin than those which occur in blood.

T1e.7 Resistance of *Trypanosoma congolense* to diminazene in mice

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In earlier work the diminazene sensitivity of *T. congolense* trypanosomes which occur in mice following treatment with diminazene has been determined. The work demonstrated that less than 0.1% of a trypanosome population relapsing following treatment is resistant to the drug dosage used and that the level of resistance expressed by such a population *in vivo* is inversely related to the population size. The work reported here has further investigated this phenomenon.

An experiment was carried out to quantify the diminazene sensitivity of two trypanosome inoculum sizes; 10^3 trypanosomes/mouse and 10^6 trypanosomes/mouse. As before, mice infected with *T. congolense* IL 3274 were treated intraperitoneally (i.p.) with diminazene aceturate at a dose of 25 mg/kg body weight (bw). Trypanosomes which thereafter appeared in the animals were collected and used to prepare the two inocula. Each inoculum was used to infect six groups of 25 mice each, via the tail vein. Immediately after infection, animals in each group were treated i.p. with the drug at a dose of 0.0, 10.0, 20.0, 30.0, 40.0 or 50.0 mg/kg bw.

Regardless of the inoculum size, trypanosomes were detected in all mice that were treated with the drug at doses of 0.0 and 10.0 mg/kg. Similarly, trypanosomes were detected in 25/25 mice that were treated with the drug at a dose of 20.0 mg/kg after infection with 10^6 trypanosomes. In the groups of mice that were infected with 10^3 trypanosomes and treated with diminazene aceturate at doses of 20.0, 30.0, 40.0 and 50.0 mg/kg bw, 15/25, 2/25, 4/25 and 2/25 mice, respectively, were detected parasitaemic. In the groups of mice that were infected with 10^6 trypanosomes and treated with diminazene aceturate at doses of 30.0, 40.0 and 50.0 mg/kg bw, 13/25, 4/25 and 2/25 mice, respectively, were detected parasitaemic. The diminazene aceturate 50% curative dose (CD50) value for mice infected with 10^6 trypanosomes (29.88 mg/kg) was significantly higher than that determined for mice infected with 10^3 trypanosomes (22.28 mg/kg).

In a second experiment, the possibility was investigated whether differences in the pharmacokinetics of diminazene with different trypanosome inoculum sizes contributed to the aforementioned results. This was investigated with three groups of mice. Group 1 comprised uninfected mice which were treated i.p. with diminazene aceturate at a dose of 25 mg/kg bw. Mice in Group 2 were infected via the tail vein with *T. congolense* IL 3274 (10^6 trypanosomes/mouse) and treated i.p. immediately after infection with diminazene aceturate at the same dosage; the trypanosomes used for infection were obtained as described for the first experiment. Mice in Group 3 were not infected but were treated intravenously, via the tail vein. Plasma samples were collected from 0–48 hours following treatment and were analysed for diminazene using high-performance liquid chromatography. A total of 23 different pharmacokinetic parameters were determined for each of the three groups. None of the parameters differed significantly between the uninfected mice that were treated i.p. and the group of *T. congolense*-infected mice that were treated i.p. It therefore appears that factors other than differences in the pharmacokinetics of diminazene are responsible for the aforementioned observations.

T1e.8 Resistance of *Trypanosoma congolense* to diminazene in goats

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In earlier work, five goats were infected with *T. congolense* IL 3274 and treated intramuscularly with diminazene aceturate at a dose of 7.0 mg/kg body weight after onset of parasitaemia; trypanosomes reappeared in all animals within ten days of treatment. When a second treatment with the same drug dosage was administered 19 days after the first, all five goats, once again, exhibited temporary remission of parasitaemia followed by a second relapse parasitaemia. Remission of parasitaemia following the second treatment could indicate that the majority of trypanosomes

arising following the first treatment are sensitive to the drug dosage used. However, the observations could also be due to either an antibody response following the first treatment, or drug accumulation. Experiments were therefore carried out to determine the validity of the latter two hypotheses. The first experiment examined the relative contributions of treatment and host antibody responses, using fluorescence activated cell sorter analysis, and used sera obtained weekly from the goats from a week prior to infection until a week after the second treatment. The results indicated that VAT-specific antibody responses did not mediate disappearance of trypanosomes following both the first and the second treatments. It therefore appeared that parasite disappearance was directly due to treatment.

In a second experiment, we endeavoured to determine whether disappearance of trypanosomes following the second treatment was associated with higher plasma concentrations of diminazene as a result of residual amounts remaining following the first. This was investigated using a series of plasma samples collected from the above five animals following both the first and the second treatments. Concentrations of diminazene in the samples were determined using high-performance liquid chromatography. Analysis of the pharmacokinetic data indicated that the area under the plasma concentration-time curve (AUC) of diminazene following the second treatment was significantly greater than that after the first dose. In addition, elimination of diminazene was significantly prolonged following the second treatment. Consistent with these results, values of the elimination half-life, mean residence time and steady-state volume of distribution of diminazene were significantly greater following the second dose than the first. However, since the C_{max} and T_{max} did not differ significantly between the two doses, it is unlikely that residual drug following the first dose accounted for differences in the drug kinetics that were observed between the two treatments. Instead, it appears more likely that the differences were due to reduced rate of elimination of the drug following treatment, which in turn resulted in increased AUC and a prolonged elimination half-life, i.e., a secondary effect of the trypanosome infection.

T1e.9 Response of diminazene-resistant and diminazene-susceptible *Trypanosoma congolense* to treatment with diminazene when occurring as a mixed infection in goats

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Earlier work has shown that in both goats and mice, the majority of *T. congolense* trypanosomes which reappear following treatment with diminazene aceturate are sensitive to the dosage that was used. Such a phenomenon could be due to the ability of sensitive trypanosomes to survive treatment when mixed with resistant trypano-

somes. The work described here was carried out to establish whether a diminazene-sensitive clone of *T. congolense* could survive treatment with diminazene aceturate when mixed with a diminazene-resistant clone in goats. Since the study used two savannah-type clones of *T. congolense*, the work necessitated the development of a polymerase chain reaction (PCR) technique to differentiate the two clones. The two clones that were used were IL 1180, a diminazene-susceptible clone, and IL 3274, a diminazene-resistant clone. The PCR technique was developed by utilizing a DNA sequence that occurs in IL 1180 but not in IL 3274. A pair of 20 bp primers (ILo 1044 and ILo 1045) were developed (by Dr. P. Majiwa, ILRAD) on the basis of DNA sequence information for the ends of the cloned DNA sequence. Using these primers in a PCR, a 900 bp sequence was amplified from IL 1180 but not IL 3274. On the basis of ethidium bromide-staining, this technique could detect 100 pg IL 1180 total genomic DNA when mixed with 25 ng of IL 3274 total genomic DNA. However, when the 900 bp product was purified and labelled with ^{32}P, the sensitivity was increased 100-fold. No 900 bp product was produced when goat blood buffy-coat DNA preparations were used directly in the PCR.

In an experiment to determine whether IL 1180 can survive treatment with diminazene aceturate when mixed with IL 3274, 24 goats were randomized into three groups of five goats each (Groups A, B and C) and three groups of three animals each (Groups D, E and F). Groups A and D were infected with IL 1180; Groups B and E were infected with IL 3274; and Groups C and F were infected with both clones simultaneously. All animals were infected intravenously, and animals that were treated were administered with diminazene aceturate intramuscularly at a dose of 7.0 mg/kg body weight (bw). Animals in Groups A and B were treated after all the goats had been detected parasitaemic. In contrast, goats in Group C were not treated until all the animals in both Groups A and B had been detected parasitaemic. Groups D, E and F served as non-treatment controls. Following treatment, all goats in all groups were monitored three times a week for 84 days for their levels of anaemia and parasitaemia. During the entire experiment, trypanosome stabilates were prepared from all animals as follows: parasitaemic mouse blood (as a result of inoculation with goat blood), once every two weeks ('M stabilates') and buffy-coat preparations of parasitaemic goat blood twice a week ('B stabilates'). All five goats infected with IL 1180 and treated with diminazene aceturate did not develop a relapse infection for the entire 84 days following treatment. This was in contrast to goats infected with IL 3274 and those infected with both clones in which five of five and four of five relapses occurred, respectively. All four of the latter animals were detected parasitaemic by 18 days following treatment. In order to determine whether IL 1180 was present in goats with mixed infections at the time of treatment (Group C), M stabilates collected three days before treatment were expanded in irradiated mice. B stabilates collected on the day of treatment were also examined, and the trypanosomes they contained examined directly. The DNA from all the aforementioned samples were then screened for the presence of IL 1180 DNA using the PCR-technique described above and the [^{32}P]-labelled 900 bp probe. For each reaction, 25 ng of genomic DNA was used as the template. The results demonstrated that IL 1180 was present in all mixed infections at the time of treatment. In order to determine whether IL 1180 was refractory to treatment when mixed with IL 3274, M stabilates of relapse trypanosome populations occurring in Group C animals on days 18, 32, 46 and 60 following

treatment were also examined. Using both of the above techniques, IL 1180 DNA could not be detected in any relapse population following treatment at the minimum level of detection (i.e., 1 pg/25 ng total genomic DNA). The data therefore indicated that IL 1180 is unable to survive treatment with diminazene when mixed with IL 3274.

T1e.10 The role of membrane transport in resistance to isometamidium in *Trypanosoma congolense*

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Samorin® (Isometamidium chloride) is a fluorophore. Interaction with intracellular components of *T. congolense* bloodstream forms (Triton X-100 solubilized lysates of clone IL 1180) was accompanied by enhancement of fluorescence. This enhancement was saturable, and titration of the fluorescence response indicated the presence of a single population of high affinity binding sites (K_d = 150–250 ng/ml). Similar enhancement developed when viable trypanosomes were exposed to the drug. These changes developed with time and were inhibited both by low temperature and by the sulphydryl-reactive compound N-ethyl maleimide. Maximal enhancement was demonstrated following permeabilization of the plasma membrane with digitonin.

In parallel experiments, uptake of ^{14}C-labelled Samorin by IL 1180 was monitored by a centrifugation assay. Uptake was linear over 15 min, and was profoundly reduced by low temperature. The time course of uptake was similar to the time course of fluorescence enhancement observed under similar conditions of temperature and drug concentration. These data indicate that Samorin enters the trypanosomal cell by a membrane-mediated transport process.

Transport processes in IL 1180 were characterized by the fluorescence enhancement and the radiolabel-uptake techniques. The concentration dependency of the initial rates of uptake of Samorin showed saturable kinetics, allowing an estimate of the K_m and V_{max} for the process. K_m was calculated to be 0.40 ± 0.10 µg/ml (n = 6) by the fluorescence enhancement method and 0.73 ± 0.35 µg/ml (n = 4) by the radiolabel uptake technique. V_{max} values from the experiments are not strictly comparable, as different properties are measured, but for the uptake of labelled drug, maximal rates were 55.5 ± 13.8 ng/min/10^8 cells.

Drug resistance appears to be a major problem with the use of Samorin in the field. Experiments were performed in order to determine the possible role of the transport mechanism in the resistance phenotype. Various populations of *T. congolense* were maintained in culture as bloodstream forms. The sensitivity of these populations to Samorin have been characterized *in vitro* [see abstract T1e.2]. Populations demonstrate a 10^3-fold variation in sensitivity. A panel of these populations was assayed for uptake using both the fluorescence and radiolabel uptake techniques. A marked correlation was noted between the uptake of drug and the degree of resistance demonstrated by the *T. congolense* populations *in vitro*. There was no consistent trend with respect to the level of resistance and the K_m of the process, but comparison of

the V_{max} value between the populations showed a clear, inverse relationship between maximal rates of uptake and resistance to Samorin.

In conclusion, these initial studies indicate that Samorin is taken up by bloodstream forms of *T. congolense* by a process that is mediated by a saturable, membrane-associated carrier system. Both the fluorescence enhancement and the radiolabel uptake techniques demonstrate similar results, indicating that both monitor the same process. Finally this process shows consistent differences between populations differing in resistance to the drug.

T1e.11 Identification and characterization of an isometamidium-binding protein in *Trypanosoma congolense*

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The aim of this work was to use the fluorescent property of isometamidium to investigate the interaction of the molecule with *T. congolense* IL 1180. We found that incubation of the compound with trypanosomes at 37 °C for 180 minutes resulted in a gradual alteration of the λ_{max} with time (from 600 nm to 584 nm) and an increase in the intensity of trypanosome-associated fluorescence of approximately two-fold. The alteration in fluorescence was temperature dependent and was inhibited by the addition of N-ethylmaleimide. In contrast, addition of digitonin to intact cells caused a rapid increase in fluorescence intensity to approximately four times that observed with intact cells. Uptake of isometamidium was also determined using radiolabelled isometamidium. The results indicated that the time course of the uptake process resembled the fluorescence profile and was temperature dependent. The results therefore indicated that the alteration in fluorescence was due to interaction of isometamidium with an intracellular component(s) and that isometamidium is transported across the plasma membrane via a protein carrier.

The above data indicated that the described fluorescence technique could be exploited to identify trypanosome proteins which interact with isometamidium causing the observed alterations in fluorescence. Scatchard analysis of triton X-100 lysates of *T. congolense* IL 1180 bloodstream forms indicated that such changes are due to interaction of isometamidium with a single intracellular molecule with a K_d of 186 ng/ml. This K_d value is very close to K_d values obtained between peptide hormones and their receptors (e.g. insulin). In additional studies we found that the level of the isometamidium-binding protein was the same in both susceptible and resistant populations of *T. congolense*. These findings were in contrast with our recent observation that there is an inverse relationship between uptake of isometamidium and the level of isometamidium resistance expressed by a trypanosome population both *in vitro* and *in vivo*.

In order to identify the isometamidium-binding protein we coupled the drug to sepharose-4B beads via cyanogen bromide. The resultant conjugate was used to produce an affinity column. Triton X-100 lysates of *T. congolense* IL 1180 were

incubated with the isometamidium-affinity column. Two protein bands of 15 and 25 kDa were identified when the column was washed with 1 mM isometamidium. This fraction caused an alteration in the fluorescence of isometamidium as previously described. Furthermore, other work has indicated that these two peptides are subunits of a single protein, since they co-purify when further purification steps are used. Since the level of the isometamidium-binding protein is independent of the level of susceptibility to isometamidium, this molecule can be used to study the mechanism of transport of the drug in both isometamidium-susceptible and -resistant populations of *T. congolense*.

T1e.12 Identification and characterization of an extrachromosomal element in *Trypanosoma brucei brucei*

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The aim of the present study was to elucidate the molecular mechanism of resistance in a multiply drug resistant *T. b. brucei* CP 547. An extrachromosomal element of 6.6 kbp was identified in undigested genomic DNA from CP 547 but not in the drug-sensitive *T. brucei* isolates tested. Higher forms (possibly multimeric forms of the 6.6 kbp element) were also identified. Attempts to isolate pure and sufficient quantities of the 6.6 kbp element by standard techniques posed many difficulties. This was overcome by generating a probe specific for the 6.6 kbp element by polymerase chain reaction (PCR) amplification using random hexanucleotide primers and the membrane-bound 6.6 kbp element as target DNA. This probe was subsequently used to screen a cDNA and a genomic λgt11 library. Sequence analysis of the positive cDNA clones revealed that two of the clones are made up of tandem arrays of a 108 bp minisatellite sequence which is flanked by 5 bp direct repeats. One fully sequenced clone has a small dimeric microsatellite made up of $(CA)_7$ at the 3-prime end of the repeat followed by a poly-A tail. Partial sequencing of five other clones has revealed that all appear to only contain tandem arrays of the 108 bp sequence. The copy number of the 108 bp repeat is variable in different *T. brucei* isolates; 2,000 copies/genome for GUTat 3.1, 6,000 for CP 547 and 8,000 for ILTat 1.1. Since the drug resistance profiles of these parasites do not correlate with the copy number of this repeat, the copy number of the 108 bp repeat is not indicative of the level of drug resistance. A puzzling result however is that drug pressure appears to select for the presence of the 6.6 kbp element, whereas it is lost if the parasites are passaged continually in the absence of drug. At present, the role of the 6.6 kbp element is unknown. Pulsed-field gel electophoretic analysis has revealed that the 108 bp sequence is dispersed throughout chromosomes of all sizes.

The 108 bp repeat is arranged in tandem arrays, is dispersed throughout the genome and occurs in a high copy number. It is, therefore, defined as a minisatellite sequence. Furthermore, since the 108 bp repeat hybridizes to chromosome-sized DNA molecules which are neither visible on ethidium bromide-stained gels nor detected by a telomeric probe, it implies that these molecules have a copy number of less than 1/genome. Interestingly, these chromosome-sized molecules were present in some

isolations of cloned populations but absent in others, indicating that these sequences are extremely active and mobile. The 108 bp minisatellite which it contains shows some characteristics of being a retroposon.

Northern blot analysis has shown that transcription of the repeat is developmentally regulated; transcripts ranging from greater than 8 kb to smaller than 1.4 kb are present in long slender and procyclic forms but are only weakly detected in short stumpy and intermediate forms.

Although it is unlikely that the 6.6 kbp element is directly associated with drug resistance, retroposons have been called 'seeds of evolution' and may be involved in genomic rearrangements leading to adaptation to environmental change and/or stress.

T1e.13 Induction and characterization of resistance to quinapyramine in a clone of *Trypanosoma congolense*

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In earlier work, resistance to quinapyramine was induced in a quinapyramine-sensitive clone of *T. congolense*, IL 1180. Studies in mice demonstrated that the 40-fold increase in resistance to quinapyramine was associated with significant cross-resistance to homidium, isometamidium and diminazene. In subsequent studies, the sensitivity of the parental clone and resistant derivative to the minimum recommended doses of quinapyramine sulphate, homidium chloride, isometamidium chloride and diminazene aceturate was determined in goats, 3, 1, 0.25 and 3.5 mg/kg, respectively. At these doses, induction of resistance to quinapyramine was associated with significant cross-resistance to homidium and diminazene, but not to isometamidium. Karyotype examination of the quinapyramine-resistant derivative and the parental clone indicated that a significant alteration in molecular weight of one chromosome occurred when the resistance was increased from 30- to 40-fold. The alteration was not observed when the resistance was increased from 0 to 30-fold, and was therefore not gradual. Comparison of trypanosome populations by polymerase chain reaction (PCR) with random primers has demonstrated differences between *T. congolense* IL 1180 and the derivative that was 40 times more resistant to quinapyramine; one primer demonstrated a difference that correlated with increased levels of resistance to quinapyramine when populations with intermediate levels of resistance were also included in the analysis. The amplified product of the PCR that correlated with increasing levels of resistance to quinapyramine was cloned into plasmid pBluescript. Two types of clones were identified; seven with an internal *Hind*III site and one without. All the clones cross-hybridized to each other. One of the seven clones (clone 2) was used to probe genomic Southern blots of sensitive, intermediate and resistant populations. The result showed that there was an increase in intensity of the sequence with increasing levels of resistance to quinapyramine. One of the clones was sequenced at both ends, but to date the sequence information does not give any clues as to what this sequence might encode. Further sequencing to complete the characterization of the PCR product is currently under way. In order to determine whether the sequence is associated with the observed alteration in

karyotype, clone 2 was used to probe a Southern blot of separated chromosomes. The probe hybridized only to a large-size class of chromosome(s). Northern blot analysis with clone 2 on sensitive and resistant parasites has been so far inconclusive.

T1e.14 Selection *in vivo* for bloodstream form *Trypanosoma brucei brucei* transfectants resistant to G418

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Recent advances in the molecular biology of *Trypanosoma brucei brucei* now allow for the genetic manipulation of these parasites by transfection techniques, predominantly in procyclic forms. Transfectants have been selected *in vitro* on the basis of resistance to aminoglycoside antibiotics, such as G418 (Geneticin), resulting from the integration by homologous recombination of a *T. brucei* gene containing the bacterial *neo* gene, encoding neomycin phosphotransferase II (NPTII). In a similar manner to procyclic trypanosomes, techniques for selection of bloodstream-form transfectants at present require growth and selection of the parasites in cultivation systems *in vitro*. Thus, the parasites need to be pre-adapted to such systems. It would be useful to select for bloodstream-form transfectants *in vivo* since most populations of *T. brucei* grow well in rodents. Therefore, the possibility of selecting transfectants *in vivo* which are resistant to G418 was tested. The pleomorphic parasite *T. brucei* GUTat 3.1, which has the capacity to undergo cyclical development, was used. Bloodstream-form GUTat 3.1 trypanosomes were transformed to procyclic cultured forms (PCFs) and cultures maintained for two months prior to their use for transfection experiments. The β-tubulin gene-targeting plasmid pUCTbneo3, digested with the restriction enzymes *Sac*I and *Sal*I to release the *T. brucei* β-tubulin fragment which contains the bacterial *neo* gene, was electroporated into GUTat 3.1 PCFs. Transfected trypanosomes were selected for by transferring the electroporated parasites to procyclic culture media containing the aminoglycoside antibiotic G418, at a concentration of 25 mg/ml. On the basis of the number of G418-resistant trypanosomes generated, the frequency of stable transfectants was estimated to be approximately 1–2 in 10^5 trypanosomes.

To establish that the G418-resistant trypanosomes arose through integration of the *neo* gene into the *T. brucei* tubulin gene cluster, parasites from the expanded cultures were harvested and embedded in 0.7% low melting point agarose and prepared for pulsed-field gel electrophoresis. The DNA was transferred to Nytran filters and the blots hybridized with a ^{32}P-labelled fragment of the *neo* gene to establish whether the *neo* gene had integrated into the *T. brucei* genome and to which chromosome it had targeted. Hybridization was observed to either one of two different sized DNA molecules of 1.5 or 2.2 megabases (Mb) for each G418-resistant clone tested, whereas no hybridization to the parental parasite was observed. In further studies the colocalization of the *T. brucei* β-tubulin genes to the 1.5 and 2.2 Mb chromosomes was determined by hybridization of ^{32}P-labelled β-tubulin to the same blot. Of ten transfectants tested, all showed localization of the *neo* gene to either of the two chromosome-sized DNA molecules without bias for either one. Colocalization of the

neo gene with the tubulin gene cluster was also established by restriction enzyme analysis and Southern blot hybridization.

Procyclic trypanosomes are not infective for mammalian hosts. However, in cultures of procyclic trypanosomes, some rodent infective forms can be generated. This was tested with cultures of one of the transfectants, GUTat 3.1/BBR3; mice became parasitaemic within ten days of infection. The presence of the *neo* gene in these parasites was confirmed by Southern blot analysis. Approximately 10^6 GUTat 3.1 parental and GUTat 3.1/BBR3 transfected bloodstream parasites were inoculated into 42 mice each which were divided into three groups of ten mice (groups I, II and III) and two groups of six mice (groups IV and V). Twenty-four hours following infection, the mice in groups I to IV were treated with G418 at doses of 10, 20, 40 and 80 mg/kg body weight (bw), respectively, by inoculating 0.2 ml of the drug in sterile water intraperitoneally. Group V comprised control, untreated mice which were inoculated with 0.2 ml of sterile water alone. Similar treatments were repeated for two additional days in all mice, resulting in three treatments per mouse. Mice were then monitored daily for 33 days for the presence of parasites by microscopic examination of wet-blood films. In the untreated control group, all mice became parasitaemic by day 2 post-infection and the first peak of parasitaemia occurred on day 14. For GUTat 3.1/BBR3, there was no significant delay in the onset of parasitaemia in any of the mice treated with G418, as compared to the control group. In contrast, with the parental clone, GUTat3.1, there was a delay in first detection of trypanosomes of three to four days for mice that became parasitaemic; at the lowest drug dose (10 mg/kg) all mice infected with the parental clone became parasitaemic whereas at 20 mg/kg, half of the mice became parasitaemic. At 40 mg/kg and above no breakthroughs were observed. We have therefore demonstrated that it is possible to select for G418-resistant transfected *T. brucei* bloodstream form parasites in mice by inoculating the drug intraperitoneally at doses between 40 and 80 mg/kg daily for three days.

T1e.15 Selection for recombination between a G418-resistant transfectant of *Trypanosoma brucei brucei* GUTat 3.1 (GUTat 3.1/BBR3) and a multidrug-resistant clone of CP 2469 (IL 3565)

Scientists: N. Murphy, S. Moloo, A. Peregrine
Technician: S. Kemei

The ability to select for *T. brucei* transfectants *in vivo* [see abstract T1e.14] offers new possibilities for studies on genetic recombination in these parasites. Since the current transfection techniques allow for the targeting of foreign genes to specific loci, genetic crosses between, for example, trypanocide-resistant field isolates and laboratory-generated transfected G418-resistant parasites are now possible. This form of selection for recombinants would therefore offer new possibilities in studies on the genetics of these organisms, particularly with regard to the identification of genes for drug resistance. Since genetic exchange has already been demonstrated for *T. brucei*, it represents a good model system for the development of systems for the selection of *T. congolense* recombinants. Two groups of sublethally irradiated rats

were infected with *T. brucei* clones GUTat 3.1/BBR3, a *neo* gene stable transfectant, resistant to G418, or IL 3565, a clone derived from a multi-drug resistant field isolate, CP 2469 (provided by Dr. Eric Zweygarth of KETRI). Since the clones have different growth rates in rodents, infections with GUTat 3.1/BBR3 were initiated three days prior to IL 3565. At peak parasitaemia the rats were killed and heparinized blood from the two groups was collected separately. Fifty ml of the infected blood from the two groups was mixed and 400 teneral male *G. m. centralis* were fed *in vitro* on the blood, maintained at 37 °C, through a silicone rubber membrane. Thereafter, the tsetse were maintained on rabbits for 27 days, left unfed for two days and on day 30 the surviving 380 tsetse were induced to probe onto warmed slides at 37 °C to identify those with mature infections. Seven tsetse (1.84%) showed metacyclics in their salivary probes. Those tsetse that were negative for metacyclics were dissected and their midguts and salivary glands were examined for trypanosomes using a phase-contrast microscope. Sixteen tsetse (4.21%) had infections only in their midguts.

The seven tsetse with mature infections were fed singly on sublethally irradiated Swiss white mice twice weekly until day 85, when all the seven infected tsetse had died. Mice were treated with diminazene at a dose of 20 mg/kg on day 1 and G418 at 40 mg/kg on days 5, 6 and 7 post-infection. Breakthrough infections were observed from four separate feedings: day 39, from flies 2, 4 and 5; day 46, from fly 5; day 53, from fly 3; and day 64, from flies 1, 2 and 5. This represents at least one breakthrough infection from five of the seven flies fed on mice. Untreated control infections were carried out on three separate occasions: day 46 when all seven flies were fed, producing seven infections; day 67 when four flies were fed, producing four infections; and day 81 when two flies were fed, producing two infections. Stabilates were prepared from all mice that became parasitaemic. Two clones have so far been generated from one of the eight breakthroughs following drug treatment. These clones, and the population from which they were derived, are currently being analysed to determine whether they are true recombinants.

T2a.1 Cloning, sequencing and expression of the gene encoding the 69-kDa trypanosome invariant antigen

Research Fellow: A. Boulangé
Scientist: E. Authié

A 69-kDa protein is a major invariant antigen in *Trypanosoma congolense*-infected cattle. The 69-kDa antigen is present in *T. congolense*, *T. brucei* and *T. vivax*, and in all stages of parasite development. A panel of mouse monoclonal antibodies (MAbs) was raised against the 69-kDa antigen. A polyclonal antibody was raised in a rabbit against the MAb affinity-purified antigen. Immunoelectron microscopy showed that the 69-kDa antigen is present in the endoplasmic reticulum and in the lysosomal compartment of the parasite.

A cDNA library constructed from *T. congolense* metacyclic polyA$^+$ RNA was immunoscreened. Seven positive clones were detected using both polyclonal antibodies and MAb 1F3. The inserts varied in size from 0.5 kbp to 1.9 kbp. They were subcloned in plasmid vector pUC 18 and sequenced. The nucleotide sequence of all clones shows a high degree of homology with the sequence reported for members of the Heat Shock Protein (HSP) 70 family. The largest insert has an open reading frame of 1.6 kbp, which corresponds to a deduced protein of 548 amino-acids. This protein shows 50–70% homology with all known HSP70; the highest homology is seen with mammalian BiP (immunoglobulin heavy-chain binding protein), and related molecules (mammalian GRP78 and *S. cerevisiae* KAR2 gene product). The trypanosome molecule differs from BiP and other related molecules by a less conserved sequence of 40 amino-acids at the C-terminus. BiP is known to be a resident of the endoplasmic reticulum, where it plays a crucial role in translocation of newly synthesized polypeptides. This is the first report that a BiP-related protein is also present in the lysosomal system, and that it acts as an immunodominant antigen.

The 1.9 kbp cDNA insert was used to probe total RNA from *T. congolense*. Two transcripts of 2.5 and 3.2 kb were detected by Northern blotting. The gene appeared to be expressed in all developmental stages of the parasite. However, mRNA levels were considerably higher in metacyclics than in other life cycle stages of the parasite. Southern blot and partial digestion analyses showed that the gene is present in the genome probably as a cluster of several copies which are not organized in tandem repeats.

A genomic library has been screened using the 1.9 kbp cDNA. The genomic clones detected will be used to determine the missing part of the protein sequence (6 kDa), the nucleotide sequence of intergenic region(s), and to identify regulatory elements.

Expression systems in *Escherichia coli* using pUR, pGEX or pMAL as vectors were tested. The ability to produce stable and soluble recombinant protein was assessed, using two cDNA inserts (1.9 kbp and 0.8 kbp). The pMAL system proved to be the most efficient. After cleavage of the carrier with factor Xa, the recombinant proteins of 63 kDa and 28 kDa were purified on MAb affinity columns. The recombinant proteins will be useful for studying the immunological responses of cattle to this molecule [see abstract T2b.6].

T2a.2 The vacuolar ATPase of *Trypanosoma congolense*

Scientist: W. Fish
Research Associates: M. Macklin, C. Wells
Technicians: C. Muriuki, D. Ndegwa

The vacuolar ATPase is an heterooligomeric enzyme involved in acidification of the endo- and exocytic pathways and in intracellular pH homeostasis. It is being investigated as an example of a potential antigen exhibiting subunits accessible via the cytosol (catalytic [A] and regulatory [B] subunits), or the flagellar pocket (proteolipid proton pore subunit or PLS).

Products from the polymerase chain reaction (PCR) of selected λgt11 cDNA clones of the catalytic (A) subunit have been sequenced and shown to include the actual 3′ end of the cDNA. These PCR results, and others, suggest that the mRNA for the A subunit in *T. congolense* is approximately 3.8 kbp with an open reading frame (ORF) of 1830 bp encoding 610 amino acids (mol. wt. = 67,635). The trypanosome subunit shows striking similarities (59 to 66%) over the entire amino acid sequence, to those of other eukaryotic A subunits while, in the central and more conserved area of the amino acid sequence, similarity approached 90%. When compared to archaebacterial and eubacterial V-type ATPases, the similarity is less (45 to 49%). At the individual level the greatest similarity is to the bovine A subunit. Some sequence motifs in the trypanosome V-ATPase are only found in archaebacterial enzymes; for example, YYRD (residues 331 to 334 in *T. congolense)* is only found in some archaebacteria, and is conserved as YFRD in all eukaryotic V- and F_0F_1-ATPases. Preliminary data from phylogeny inference computer programs (PHYLIP) show that, these sequence similarities notwithstanding, the trypanosome A subunit aligns independently from archaebacterial and eukaryotic A subunits, whether compared to each group separately, or together. More exhaustive comparisons may yield sequences worthy of examination as potential defined antigens. As previously reported, a 1.4 kbp fragment of an internal ORF has been successfully inserted into the pAX4b expression vector for assessment of the expressed protein as an immunogen.

Most restriction enzyme subclones from cDNA of the *T. congolense* B (regulatory) subunit have been completely sequenced. Although the ORF lacks 30 amino acids, the data has confirmed regions of the sequence necessary to successfully clone a fragment(s) into expression vectors.

PCR has not yielded products for the membrane-bound proteolipid proton pore subunit (PLS), even though many redundant primers were tried, some designed with trypanosome codon usage bias. cDNAs for this subunit were kindly supplied by others—*Drosophila melanogaster* and *Nephrops norvegicus*, M.E. Finbow, Beatson Institute, Glasgow, UK; *Torpedo marmata*, F.-M. Meunier, CNRS, Gif-sur-Yvette, France; and *Bos taurus*, N. Nelson, Roche, USA. The latter two cDNA inserts have been used for screening a *T. congolense* metacyclic cDNA library. That of *T. marmata* gave no positive signals, while the bovine PLS cDNA has given weak signals and is now being re-examined under conditions of different stringency.

Given the remarkable conservation at the amino acid level (67 to 80% over the ORF) of the PLS, a relatively small molecule (17 kDa), the lack of strong positive

signals is surprising. It is possible that the bias of codon usage seen in trypanosomes is a contributing factor, and could also explain the lack of success seen with the PCR. Alternatively, it is possible that trypanosomes possess a proteolipid pore subunit of significantly different sequence, or may use that of another enzyme, for example the F_0F_1-ATPase.

T2a.3 The GTP-binding proteins of African trypanosomes

Scientists: D. Grab, D. Russo, W. Fish
Technician: Y. Verjee

In the bloodstream of the mammalian host, *Trypanosoma brucei* differentiates, via intermediate forms, from rapidly dividing slender forms into non-dividing stumpy parasites. Western blot analysis using antibodies against $G_{o\alpha}$, transducin (G_{i2}) and G_β subunits indicates the existence of several G-proteins in both slender and stumpy form parasites. In rat-adapted *T. brucei*, two major proteins with Mrs of 53 and 64 kDa are recognized in stumpy form parasites by anti-G_β antibody. In contrast, this antibody recognized two proteins of 54 and 58 kDa which were present in slender form parasites. The major protein recognized by anti-transducin antibody is a 72-kDa protein. This protein is predominant in stumpy but not in slender forms of rat-adapted *T. brucei* parasites. The 72-kDa protein was also expressed in bloodstream forms of *T. brucei* cultivated *in vitro* as well as in promastigote and epimastigote forms of *T. congolense*. Little or no 72-kDa protein was detected in *T. congolense* bloodstream or metacyclic form parasites. When the blots were probed with anti $G_{o\alpha}$, a major 53-kDa protein is detected in rat-adapted *T. brucei* slender bloodstream forms. However, in stumpy parasites the same antibody reacts strongly with a 64-kDa protein, whilst proteins with Mrs of 43, 52, 58 and 160 kDa are also recognized.

Using the [α-^{32}P]-GTP-binding assay of Bhullar and Haslam *(Biochem. J.*, 1987, 245: 617) the presence of a 27-kDa high affinity Mg^{2+}-dependent protein in *T. congolense* has been demonstrated. This protein was present in higher amounts in promastigote, epimastigote and metacyclic forms (present in the tsetse fly) than in bloodstream forms of the parasite. The binding of [α-^{32}P]-GTP was inhibited by both EDTA and GTP, and partially inhibited by ATP.

T2a.4 The transferrin cycle in African trypanosomes

Scientists: D. Grab, D. Russo, J. Lonsdale-Eccles, J. Naessens, M. Shaw, P. Webster*
Research Associates: C. Wells, G. Mpimbaza
Technician: Y. Verjee

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The cellular organelles of *T. congolense* and *T. brucei* which label with anti-transferrin (Tf)IgG, as well as with endocytosed Tf-Au_5, are similar to those which have been

shown to contain endocytosed Tf-Au_{15} complexes. Furthermore, in *T. congolense* it has been shown that Tf colocalized with a lysosomal marker. These data strongly suggest that African trypanosomes, although able to endocytose Tf in a receptor-mediated manner, may not recycle Tf, but rather deliver the ligand to the lysosome. In view of these findings, it may be possible to covalently bind drugs and plant or bacterial toxins to Tf, as has been described for immunotoxins, for use as a chemotherapeutic agent. We are currently attempting the feasibility of the latter by directly coupling anti-trypanocidal drugs onto Tf.

Further, the presence of Tf-binding proteins in Triton X-100 lysates of *T. brucei* have been demonstrated. Unlike Schell *et al. (EMBO J.* 10: 1061, 1990), we found a Tf-binding protein which appears to have properties similar to the mammalian Tf receptor. The molecule, as analysed by Sephacryl S-300 is capable of binding one molecule of $diFe^{3+}$. The pI of the Tf-binding protein (pI 5.4-5.8) is similar to the mammalian Tf-receptor. The pH of the trypanosome endosome may be sufficiently low to release both Fe^{3+} and Tf from the trypanosome receptor. We were able to block trypanosome growth with an antiserum (and Ig purified from it) made to a protein fraction containing Tf-binding molecules and this antiserum recognized a 90-kDa protein on Western blots. However, monoclonal antibodies will be necessary to show whether the 90-kDa Tf receptor-like protein is the target for blocking of trypanosome growth. Such a protein could well be a vaccine candidate.

T2a.5 Trypanosomal proteases

Scientists: J. Lonsdale-Eccles, D. Grab, W. Fish, E. Authié
Research Associates: G. Mpimbaza, C. Wells
Research Fellow: D. Russo
Technicians: D. Ndegwa, C. Muriuki

It has been shown that there are several dissimilar types of protease activity in African trypanosomes (Kornblatt *et al. Arch. Biochem. Biophys.*, 1992, 293: 25–31; Mbawa *et al., Eur. J. Biochem.*, 1992, 204: 371–379). Each of these may play discrete roles in the metabolism of different life cycle stages of the parasite (Mbawa *et al., Eur. J. Biochem.*, 1991, 195: 183–190) and appear to be subject to unusual control mechanisms (Lonsdale-Eccles and Grab, *Eur. J. Biochem.*, 1987, 169: 467–475).

While dissecting the complex endocytic-lysosomal system of African trypanosomes by temperature, protease-inhibitor and membrane perturbations, it was observed that proteases may play key roles in the degradation of anti-variable surface glycoprotein antibodies. Specific protease inhibitors limited the internalization of the antibodies by the parasites and intralysosomal degradation was markedly diminished. Thus, the appropriate use of proteases inhibitors should permit the dissection of this important defense mechanism by the parasites against the host's antibody response.

Biochemical analyses of the major 33-kDa antigen of *Trypanosoma congolense,* for which antibody production is correlated with enhanced resistance to trypanosomiasis, has shown that the 33-kDa antigen (Authié *et al., Mol. Biochem. Parasitol.*, 1992, 56: 103–116) is a previously characterized trypanosomal cysteine protease (Mbawa *et al., Eur J. Cell Biol.*, 1991, 56: 243–250; Mbawa *et al., Eur.,*

J. Biochem., 1992, 204: 371–379). As postulated previously, this suggests that the enzyme is released into the host where it may play an important role in the pathology of trypanosomiasis. Successful elimination of the protease from the circulation by antibodies may minimize host pathology caused by these degradative enzymes. It also raises the possibility that these enzymes may prove useful as specific indicators of infection, because each of the proteases of the different trypanosome species is distinct (Mbawa *et al. Eur. J. Biochem.*, 1991, 195: 183–190).

Peptidyl diazomethyl ketones (donated by Dr. Elliot Shaw, Switzerland) have been shown to be highly effective inhibitors of several of these intracellular enzymes and have also been shown to be toxic to the parasites *in vitro* (Mbawa *et al., Eur. J. Biochem.*, 1992, 204: 371–379). This raises the possibility that such inhibitors may prove useful as drugs to kill parasites or to minimize host pathology.

T2a.6 Characterization of casein kinase II of *Trypanosoma congolense*

Student: R. Janoo
Supervisor: O. ole-MoiYoi
Co-supervisors: P. Majiwa, N. Murphy

Casein kinase II (CKII) is a serine/threonine-specific protein kinase that is messenger independent and that has as its physiological substrates a wide variety of proteins involved in diverse cellular processes. The phosphorylated residues are usually N-terminal to a cluster of acidic amino acid residues. Recent studies have shown that an increasing number of these protein substrates are phosphorylated at site motifs specific for CKII. Included in this group are eukaryotic and viral proteins that regulate cell growth, differentiation and transformation.

Analysis of CKII from a variety of organisms including *Theileria parva, Caenorhabditis elegans, Drosophila melanogaster, Zea mays,* yeast and mammals reveals a remarkable degree of sequence conservation. Polyclonal antibodies to mammalian CK II have thus reacted to CK preparations from all these organisms. Homogenates from *T. brucei,* or partially purified preparations of CK II from this organism, revealed no reactivity with anti-bovine CK II IgG. Furthermore this immunological distinctiveness is supported by functional data showing that effector molecules such as polyamines or glycosaminoglycans do not have the expected effects on trypanosome enzymes.

CKII from *T. congolense* IL 3000 has been isolated and partially purified using DEAE-cellulose, ion-exchange chromatography with a specific activity of 88,997 units/ug and a purification factor of 20-fold, and heparin-agarose/Sepharose, affinity chromatography with a specific activity of 270,967 units/ug and a purification factor of 61-fold. Amino acid sequence analysis of this partially purified enzyme will enable us to better define how different the mammalian and parasite casein kinase-like enzymes are, and this may be extended to differences in functional activities including susceptibility to stimulation or inactivation by a variety of effector molecules, including anti-trypanosomal drugs.

Conserved domains of CK II at the amino acid level have been identified by comparison with published enzyme sequences. This information has been used to construct oligonucleotides for use in the polymerase chain reaction (PCR). Using *T. congolense* IL 3000 genomic DNA, these primers have enabled the amplification of sequences of about 550 bp. The PCR products will be cloned with appropriate vectors for antigen expression and for the generation of antibodies.

T2a.7 Possible chaperone-like molecules of African trypanosomes

Scientists: D. Nandan, E. Authié, T. Pearson*
Research Associate: C. Wells
Technician: D. Ndegwa

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Whilst the function of some stress proteins are unknown, many heat shock proteins (HSP) are known to play a role in molecular chaperoning. A simple one-step procedure using gelatin-agarose has been used for the purification of two constitutively expressed molecular chaperones from African trypanosomes. The molecular masses of the proteins are 68 kDa and 72 kDa respectively. The 68-kDa protein reacted with a monoclonal antibody which also reacts with members of a family of 70-kDa HSPs 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* [see abstract T2a.1].

A reproducible amino-terminal sequence for the 72-kDa protein has been obtained but demonstrated no significant homology to any other known protein. The amino terminus of the intact 68-kDa polypeptide is apparently blocked from sequence analysis. Attempts are being made to obtain proteolytic fragments of the 68-kDa protein for microsequencing. The amino acid composition of both the proteins has been obtained and analysis of both proteins by two-dimensional gel electrophoresis has revealed their acidic nature; the pI of major stress proteins is in the acidic range.

Cell fractionation suggests that the 68- and 72-kDa proteins have cytosolic and membrane localizations, respectively. Immunoelectron microscopy using rat polyclonal sera raised against the 72-kDa protein showed clear localization in endoplasmic reticulum and lysosomal-like organelles in trypanosomes. Membranes were isolated from *T. vivax* and treated with trypsin and V8 protease in the presence and absence of detergent and the relative levels of 72 kDa were examined by Western blot analysis. Results showed the inaccessibility of the 72-kDa protein to proteolysis and suggest its lumenal localization.

In order to study the developmental regulation of trypanosome chaperones, the level of the 68- and 72-kDa proteins were monitored as bloodstream forms of *T. congolense* IL 3000 transformed into procyclic culture forms. The samples were taken at various intervals after initiation of transformation *in vitro*. The status of transformation was checked by the appearance of procyclin on the surface of

transforming parasites. The results showed consistent increases in the level of both presumptive chaperones and suggest a role for these chaperones during development.

T2a.8 Identification and characterization of invariant surface molecules of African trypanosomes

Scientists: D. Nandan, J. Lonsdale-Eccles, T. Pearson*
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Reports regarding cell surface receptors on trypanosomes indicate their low abundance. Various approaches have been used to identify cell surface molecules, including the use of various plant lectins. Biotinylated *Ricinus communis* Agglutinin I (RCA) has been employed in combination with streptavidin-agarose to purify trypanosome membrane glycoproteins containing galactose as a constituent of their oligosaccharide chains. Under the purification conditions used, very little VSG, if any, copurified with RCA-binding proteins. The mixture of RCA-binding proteins were used to raise monoclonal antibodies in mice. Western blot assays using MAbs DN 33.9 and DN 33.11 revealed reactivity with a 100-kDa protein. Other antibodies, MAbs DN 45.2 and DN 63.5, recognize 72- and 43-kDa proteins, respectively. Further characterization of MAb DN 33.9 showed that this 100-kDa protein is a membrane protein and is present in all the life cycle stages of *T. congolense* IL 3000. The absence of cross reactivity with bovine liver proteins suggests that the protein is parasite-specific. Studies will continue to assess the surface exposure of the RCA-binding proteins.

The presence of a mannose-6-phosphate receptor on eukaryotic cell surfaces and its involvement in the process of endocytosis are well documented. β-glucoronidase is a lysosomal enzyme containing phosphomanan as a constituent of its oligosaccharide chain. β-glucoronidase was used in ligand affinity chromatography to identify and purify mannose-6-phosphate binding protein(s) from trypanosomes. Crude trypanosome membranes were used as the starting material for the purification of mannose-6-phosphate binding proteins(s). The detergent solubilized membrane proteins were incubated with β-glucoronidase-agarose for two hours at 4 °C, washed with buffers containing detergent, and finally with buffer supplemented with glucose-6-phosphate. The remaining bound proteins were eluted with mannose-6-phosphate and concentrated and separated by SDS-PAGE. Silver staining of SDS-PAGE gels revealed a closely spaced triplet of polypeptides in the region of 40 kDa. Mannose-6-phosphate binding proteins recovered from trypanosomes labelled with ^{35}S-methionine also migrated in the region of 40 kDa on SDS-PAGE. Results of both these experiments demonstrate the presence of mannose-6-phosphate binding proteins in trypanosomes.

T2a.9 Phosphatases of *Trypanosoma congolense*

Research Fellow: T. Omalokoho
Supervisors: J. Lonsdale-Eccles, D. Grab

Lysates of *Trypanosoma congolense* IL 3000 bloodstream forms were obtained by passing the parasites through a French pressure cell at 1500 psi. The post-nuclear supernatant of the lysate, obtained by centrifugation of the lysate (1,700 × g; 10 min), was further centrifuged (13,500 × g; 60 min) to yield a high speed supernatant (HSS) and a high speed pellet (HSP). The fractions were assayed for acid phosphatase activity using *p*-nitrophenyl phosphate (10 mM) at 37 °C, pH 5.0 (50 mM sodium acetate). The released *p*-nitrophenol was measured at 405 nm after the addition of 4 vols 10 mM NaOH. The HSS contained about 20% of the total lysate enzyme activity while a 3% Triton X-114 detergent extract of the HSP yielded about 55% of the activity.

Chromatography of the HSS and the detergent extract of HSP were performed on a column of Sephacryl S-300 from which the native molecular masses of the individual acid phosphatase activities were determined. HSS yielded two peaks of activity (AcP_1 and AcP_2), while the detergent extract yielded a single peak (AcP_3). Further enzyme purification was performed by chromatography on DEAE-cellulose and by isoelectric focusing. The three enzyme activities could be distinguished on the basis of Mr, pI, their affinities for the substrates *p*-nitrophenyl phosphate and β-glycerol phosphate, and extent of inhibition by sodium fluoride. Monoclonal antibodies are being raised to the respective enzymes to examine their intracellular location and the possible functional differences between the three distinct activities.

T2b.1 Development of bovine cytokine reagents for the study of T/B-cell responses

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An expansion of the $CD5^+$ B-cell subpopulation in *Trypanosoma congolense*-infected cattle has been observed. The cause of this increase in $CD5^+$ B cells is not known and might be due to particular stimuli. To study B-cell activation, bovine cytokines, and in particular growth factors like IL-4, are required. IL-4 is a T cell-derived lymphokine which is involved in regulating B-cell differentiation and Ig-isotype switch. A full-length bovine IL-4 cDNA probe was kindly provided by D. Dobbelaere. For the expression of IL-4 in *Escherichia coli*, several recombinant plasmids were constructed. A *Nco*I-*Ssp*I cDNA fragment coding for full-length mature bovine IL-4 (113 amino acids), and an *Eco*RI-*Rsa*I fragment coding for 70 amino acids of the protein were sub-cloned into the expression vector pGEX-3X. The correct insertion was shown by sequence analysis. Fusion proteins were purified under non-denaturing conditions at a yield of 3 mg/l of bacterial culture.

On SDS-PAGE, the full-length fusion protein, fIL-4-GST, has an Mr of 40 kDa, the partial IL-4 fusion product, paIL-4-GST, has an Mr of 35.5 kDa. The glutathione-S-transferase carrier was removed by cleavage with factor Xa resulting in protein bands with Mrs of 27 kDa (GST), 13.5 kDa (full-length IL-4), and approximately 8 kDa (partial IL-4). The fusion proteins and Xa cleavage products are being tested for biological activity.

The recombinant proteins, either the fusion protein or the Xa-cleaved IL-4 product were used as immunogens to raise polyclonal antibodies in mice and rabbits. The specificity of the mouse antisera was confirmed by immunoblotting on semi-purified recombinant IL-4 proteins. Monoclonal antibodies to the recombinant protein are also being generated.

Other bovine cytokine reagents important for the study of T/B-cell responses, IL-1α, IL-1β, IL-2 and IL-6, have been obtained from other sources. Some of the reagents were sub-cloned in riboprobe vectors. DNA probes, riboprobes and a set of specific primers for each cytokine have been assembled at ILRAD.

T2b.2 Antibody recognition of transfected bovine gene products from mouse cells

Scientists: J. Naessens, M. Sileghem, N. McHugh, P. Toye, W. Davis*, R. Reeves*, D. Dobbelaere†
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The cDNA encoding the bovine IL-2 receptor α chain (IL-2Rα) has been transfected into mouse L fibroblasts and stable transfectants selected using a rabbit antiserum against a fusion protein of the gene. A panel of monoclonal antibodies (MAbs) to bovine activation antigens have been screened by identifying antibodies which bound to transfected but not untransfected cells. Three MAbs (one from ILRAD and two from Washington State University) were selected on this basis and all three precipitated a molecule of Mr 55 kDa under reducing conditions from activated cells. The precipitated molecule is of the expected size considering homologies with mouse and human IL-2Rα (CD25, Tac). One of the three MAbs was a strong inhibitor of IL-2-dependent proliferation of bovine lymphocytes. Thus, the availability of transfected cells allowed a direct method of establishing the specificity of antibodies to bovine activation antigens.

T2b.3 Characterization of transferrin receptor

Scientists: J. Naessens, D. Grab
Technician: J. Nthale

A mouse IgM monoclonal antibody (MAb), IL-A77, has been produced which is specific for the bovine transferrin receptor. The antigen recognized by the MAb is present on immature erythroid cells and proliferating lymphocytes, and it appears on resting cells within 24 hours after stimulation with Concanavalin A or pokeweed mitogen. The MAb could block the binding of radiolabelled transferrin to its cell surface receptor. Immunoprecipitation showed that the antigen is a disulphide-bonded dimer of two identical chains of Mr 90 kDa. Under non-reducing conditions it migrated as a 190-kDa protein in SDS electrophoresis, but it showed as one band of 90 kDa after reduction. The transferrin could also be isolated in a relatively pure form by binding a cell lysate to immobilized transferrin, washing in desferal and eluting with a neutral buffer containing 1M KCl. A 90-kDa protein was recovered, which could be precipitated by IL-A77.

Since activated and transformed B cells express large quantities of transferrin receptor, it may be a good marker for measuring B-cell activation after 12 hours.

T2b.4 Lack of IgD on ruminant B lymphocytes

Scientist: J. Naessens
Technician: J. Nthale

There have been speculations in literature that IgD has a role in activating/suppressing the B-cell response. However, no evidence has been found for a second heavy chain class on the surface of peripheral blood, lymph node or spleen B cells from cattle. Precipitation with a monoclonal antibody to Ig light chain revealed only one heavy (μ) chain. Preabsorption of a B-cell lysate with anti-μ did not leave additional Ig light chains. Since a second class could have been missed because of abnormalities/peculiarities in the specificities of the anti-μ and anti-light chain monoclonals, the same precipitations were repeated on sheep cells, with a different set of monoclonals, and no evidence was again found for an IgD class. This is in contrast to other species for which reports have described the existence of IgD (chicken, rabbits, rodents, primates). Thus B-cell activation in ruminants may be initiated and/or progresses in a different manner from that in other species, or that the normal B cells in adult ruminants belong to a different type/lineage of B cells.

T2b.5 Homology between bovine and human V_H genes

Scientists: J. Naessens, M. Schutte*

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Hybridizations with probes specific for human V_H gene families revealed more than 15 bands for the V_H1 and V_H3 gene families, and ten bands for the V_H4 gene family, but none with probes from the small V_H2, V_H5 and V_H6 gene families using bovine genomic DNA in Southern blots. This is in agreement with homologies between mouse and human V_H genes.

A cDNA λgt11 library was constructed from Concanavalin A and phorbol ester-stimulated bovine lymphocytes, and was screened with human V_H1, V_H3 and V_H4 probes and a cocktail of probes of all 6 V_H gene families. No positive colonies out of 500,000 p.f.u. were observed under low and very low stringencies.

The polymerase chain reaction (PCR) was used in attempts to isolate bovine V_H gene segments. Primers were designed to specifically anneal to human V_H regions or to a consensus sequence of human and murine V_H regions. Although several PCR fragments of the correct size were obtained, sequencing revealed that none were V_H gene fragments.

T2b.6 69-kDa BiP homologous protein

Scientist: E. Authié
Research Fellow: A. Boulangé
Technician: D. Muteti

The immunodominant 69-kDa antigen of *T. congolense* has been identified as an HSP70, homologous to mammalian BiP (immunoglobulin heavy chain binding protein) [see abstract T2a.1]. Native 69-kDa protein remains difficult to purify. This is in part due to its BiP-related ability to bind a wide range of proteins. Recombinant proteins of 63 kDa (r63) and 28 kDa (r28) were produced in the pMAL expression system in *E. coli* and purified on monoclonal antibody affinity columns.

Antibody responses to the native 69-kDa molecule showed differences between N'Dama and Boran cattle, the former having higher titres of IgG_1 than the latter in ELISA. However, the responses in the two groups were similar when r63 was used as the antigen. When sera from infected cattle were absorbed with r28, reactivity to r63 was completely abrogated, thus demonstrating that all the epitopes recognized on the r63 antigen are located on the C-terminal half of the molecule. However, sera from infected N'Dama cattle absorbed with r63 or r28 were still able to recognize epitopes on the native molecule, whereas the absorption depleted most of the reactivity to the 69-kDa antigen in the sera of Boran cattle. Thus antibody responses in N'Dama and Boran cattle may not only differ in isotypic and quantitative aspects but may also be different in the nature of epitopes recognized.

T-cell proliferation assays were carried out using both native protein and r63. Native protein did not elicit significant proliferative responses in peripheral blood mononuclear cells from primary infected N'Dama and Boran cattle. However, proliferative responses to r63 (0.2–5 µg/ml) were detected as early as one week after infection in two out of the four N'Damas (ND40 and ND41). All eight cattle became responsive to r63 during the following six weeks, but there were wide fluctuations within individuals over time.

T2b.7 Immune responses to *Trypanosoma congolense* cysteine protease (congopain)

Scientists: E. Authié, G. Duvallet*
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The levels of anti-congopain IgG were measured in five breeds of cattle of differing susceptibility to trypanosomiasis during experimental primary infection with *T. congolense*. The two trypanotolerant breeds had high antibody levels, whereas the three susceptible breeds exhibited low levels. Moreover, N'Dama × Boran crossbred cattle, of intermediate susceptibility compared to the parental breeds, had intermediate levels of antibodies. We extended these observations to trypanotolerant Baoulé and susceptible Zebu cattle exposed to natural tsetse challenge, under conditions

where Zebu but not Baoulé cattle were repeatedly treated. Zebu and Baoulé cattle had similar levels of anti-congopain antibodies. This result confirmed that congopain is antigenic in all cattle and demonstrated that repeated infections terminated by drug therapy in Zebu stimulate antibody responses of similar levels to those generated in untreated taurine cattle.

Proliferative responses of total peripheral blood mononuclear cells to congopain were studied in primary infected N'Dama and Boran cattle. No response was detected in either breed using native, i.e. active, protease. However, proliferative responses were detected in both groups of cattle when heat-inactivated congopain (10 µg/ml) was used as the antigen. The response was detected as early as one week after infection in two out of four N'Damas.

T2b.8 Immunosuppressive potential of congopain

Scientist: E. Authié
Research Associate: K. Taylor
Technician: K. Tikolo

Heat-inactivated congopain antigen, but not the active enzyme, elicited proliferative responses of peripheral blood mononuclear cells (PBMC) from infected cattle, suggesting a possible immunosuppressive activity of congopain. The suppressive effect of congopain on responses of normal bovine PBMC to Concanavalin (Con A) and to Foot and Mouth Disease virus antigen (FMDV) was investigated.

Affinity purified congopain (0.1 to 10 µg/ml) was co-incubated with PBMC and either Con A (5 µg/ml) or FMDV. In cultures incubated for 48 hours or more with congopain at concentrations > 1 µg/ml, incorporation of ^{125}I-Iodo-deoxyuridine by Con A-stimulated PBMC was reduced by 10–30% and IUdr incorporation of FMDV-stimulated PBMC was reduced by 50–60%. Both effects were dependent on the dose of congopain used and on the duration of co-incubation. The suppression was not due to direct cytotoxicity of congopain. The suppression was abrogated by heat-inactivation or iodoacetamide alkylation of the protease. The suppression was not due to consumption of essential medium nutrients, nor to a direct effect on mitogen/antigen. Expression of IL-2 receptors on lymphocytes incubated in the presence of congopain was not altered. The effect of congopain on IL-2 secretion and on interferon production is still to be investigated.

The immunosuppressive effect of congopain occurred despite the presence in culture medium of high amounts of alpha-2-macroglobulin (A2M), a major inhibitor of proteases. Bovine A2M has been purified. A shift in the molecular weight of A2M from 185 kDa (native form or 'slow form') to 85 kDa ('fast form') occurs in the presence of active congopain, thus demonstrating that the trypanosome protease interacts with A2M. 'Activated' A2M, i.e. the complex between A2M and proteases, has been shown to have immunosuppressive effects. It is possible that the suppressive effect of congopain on proliferation of bovine PBMC to mitogens or antigen is mediated by the protease-A2M complex.

T2b.9 Antibody responses to variable surface glycoproteins in *Trypanosoma congolense*-infected N'Dama and Boran cattle

Scientist: D. Williams
Research Associate: K. Taylor
Technician: B. Gichuki

Antibody responses to surface-exposed epitopes (SEE) of variable surface glycoproteins (VSG) were characterized in Boran (susceptible) and N'Dama (trypanotolerant) cattle following primary *T. congolense* infection. Using a complement lysis assay (CLA) initial data from a comparison of three N'Damas and three Borans suggested that higher levels of serum antibody were produced in the N'Damas. However when these studies were extended to a further eight N'Dama and eight Borans, overall no significant difference was observed between the two breeds in the level of antibody to SEE of VSG.

Three clones of parasites each expressing a different VSG were isolated from three animals (two N'Damas and one Boran) 14, 25 and 35 days after infection. These clones together with the infecting clone (IL 1180) were tested in the CLA to determine if N'Damas produced an antibody response to more of the VATs expressed during infection than the Borans. However there was no difference between N'Damas and Borans in the recognition of different variants expressed during infection. These results suggest that quantitative differences in the antibody response to SEE of VSG do not contribute to the N'Damas superior ability to control the disease.

The isotype of antibody recognizing SEE of VSG was determined by fluorescence activated cell sorter analysis, although this assay was not quantitative. Anti-SEE antibodies of the IgM, IgG_1 and IgG_2 isotypes were detected. A study was also carried out to characterize the levels and isotype of serum antibodies to internal VSG epitopes using formalin-fixed trypanosomes in an ELISA. There was no difference between N'Damas and Borans in the levels of IgM detected in this assay. However, significant differences were observed in the IgG_1 titres. Higher IgG_1 titres were consistently observed in the N'Damas from day 28 onwards. These data support the observations made previously which identified differential isotype responses to a trypanosome cysteine protease and a 69-kDa heat shock protein [see abstracts T2b.5 and T2b.6]. No IgG_2 antibodies against internal VSG epitopes have so far been detected.

These results suggest that in Boran cattle either more IgG_1 antibodies are absorbed from the circulation or that there is an inhibition in the switch from IgM to IgG_1 during *T. congolense* infections. Thus, assays are being developed to measure the number of antibody-secreting cells and the isotype of the antibody they secrete in N'Dama and Boran cattle during infection.

T2b.10 Cellular responses to variable surface glycoproteins in *Trypanosoma congolense*-infected N'Dama and Boran cattle

Scientist: D. Williams, J. Naessens
Research Associate: K. Taylor
Technician: B. Gichuki

Data from *T. b. brucei*-infected mice suggest that the antibody response to variable surface glycoprotein (VSG) can be controlled in a T-independent manner. However, few data are available to show that this situation exists in cattle. Thus, spleen cells and peripheral blood mononuclear cells (PBMC) were collected from a Boran calf immunized with irradiated trypanosomes and from two Boran and two N'Dama steers following *T. congolense* infection. The cells were cultured for five days with either purified VSG, Foot and Mouth Disease virus antigen (FMDV) or Concanavalin A (Con A). Cell proliferation was measured by IUdr incorporation. Cells from all five animals responded to both FMDV and Con A but no VSG-specific response was observed at anytime.

These data suggest either that (1) there is no VSG-specific T-cell response in peripheral blood or spleen, (2) the responding cells are too few to be detected in the assay, or (3) that the VSG had been prepared in such a way that it had lost its antigenicity. All five cattle had a detectable antibody response to VSG.

T2c.1 Cloning and expression of bovine tumour necrosis factor α

Research Associate: L. Gaidulis
Scientists: O. ole-MoiYoi, M. Sileghem

To study the expression of tumour necrosis factor α (TNFα) gene, probes have been prepared. Two sets oligonucleotides (outer and nested) were designed with sequences based on the conserved nucleotide segments of known mammalian TNFs. Such oligonucleotides were used as primers in a polymerase chain reaction (PCR). In a first approach, mRNA was isolated from monocytes arbitrarily stimulated with phorbol myristate acetate and cDNA prepared. The outer and nested primers were used to amplify a 393 bp and a 344 bp product, respectively, which proved to be partial sequences from TNFα. In a second approach, a new set of oligonucleotide primers amplified a 570 bp product by PCR from the TNFα-secreting cells, whereas no product was amplified from the same cells under conditions where no TNFα was produced. The amplified product was subcloned into Bluescript and sequenced. The full sequence of the TNFα message was shown to have an 80–90 % homology to other mammalian TNFα sequences. The incomplete 344 bp sequence was used to generate a riboprobe which was used to detect mRNA expression. In Northern blots a signal was detected in RNA from lipopolysaccharide-stimulated alveolar macrophages but not in the RNA from unstimulated alveolar macrophages. Using PCR to detect cytokine mRNA expression, a signal was detected in both unstimulated and stimulated cells. To attempt semi-quantitative titrations using the PCR methodology, primers for bovine β actin were synthesized and used as a control with primers for bovine TNFα in a double amplification PCR. However, although a product was detected, actin RNA levels are also elevated in stimulated cells so that constitutive expression of a further housekeeping gene is being explored for control of the TNFα assay.

The full length 574 bp TNFα cDNA coding region of both N'Dama and Boran cattle has been cloned and sequenced. Sequence differences observed between the two breeds will be further studied to determine if they represent PCR errors or actual polymorphisms. Work is presently in progress to express TNFα in *E. coli* to produce bulk quantities for biological studies.

T2c.2 Immunological studies of bovine tumour necrosis factor α

Scientists: M. Sileghem, L. Logan-Henfrey, E. Authié
Technician: R. Saya, K. Tikolo

A sandwich ELISA specific for bovine tumour necrosis factor α (TNFα) was developed using a monoclonal antibody and a polyclonal antiserum. Initially, a polyclonal antiserum provided by Dr. J. Ellis was used. Later, a polyclonal antiserum produced at ILRAD was used. Plasma samples from animals infected with *T. vivax* IL 2337 (provided by Dr. L. Logan-Henfrey) were screened with this ELISA. Activity was detected in samples harvested two weeks after infection. Although this activity was above the limit of detection, it was very low and it was not clear whether it actually represented the presence of TNFα. Therefore, an alternative strategy was

used where monocytes were purified and cultured *in vitro;* the culture supernatants were then screened for TNFα. Using this system, a marked TNFα secretion was noted which, in terms of kinetics, matched the pattern obtained from the plasma samples. In contrast to the strong TNFα production seen with monocytes from *T. vivax* IL 2337-infected animals, no activity was seen with monocytes from *T. congolense* ILNat 3.1-infected animals.

The ELISA system was also used to study production of TNFα by bovine monocytes following stimulation *in vitro*. Bacterial lipopolysaccharide (LPS) and interferon γ (IFNγ) were both found to be poor stimulators. However, when the cells were cultured with IFNγ for 18 hours and then triggered with LPS for five hours, a potent TNFα response was noted. Thus, two types of activating factors seem to be required for optimal TNFα secretion, a priming factor and a trigger factor. A *T. congolense* lysate was found to be able to substitute the trigger but not the primer. Minor contaminations with bacterial endotoxins were found to affect the assay and addition of 500 U/ml polymyxin B was routinely used to reduce false positives as a consequence of endotoxin contamination. The stimulation of TNFα secretion *in vitro* was also used to generate clear cut positive and negative controls for the technological research on TNFα. Using such controls, intracellular TNFα was detected using flow cytometry. Studies will continue to determine whether specific antigens such as the 33-kDa cysteine protease and the 69-kDa heat shock protein can stimulate the production of TNFα in this *in vitro* system.

T2c.3 Uptake of trypanosomes by monocytes from Boran and N'Dama cattle throughout infection with *Trypanosoma congolense*

Scientist: M. Sileghem
Technician: R. Saya

A two-colour flow cytometric analysis was developed to follow uptake of trypanosomes by bovine monocytes throughout infection. Firstly, a polyclonal bovine antiserum specific for *T. congolense* IL 1180 was generated through injection of irradiated parasites. Binding of IgG and IgM to living parasites was studied by flow cytometry and agglutination. Secondly, the parasites were labelled with fluorescein (green fluorescence), opsonized and incubated with peripheral blood mononuclear cells (PBMC) at 37 °C. To avoid loss of cells by adherence, gelatin-coated tubes were used. Finally the macrophages were stained with a monoclonal antibody conjugated to phycoerythrin (red fluorescence) at the end of the incubation. Using a two-colour cytometric analysis, it was found that many trypanosomes were bound by monocytes but none by lymphocytes and that binding was strictly dependent upon the presence of opsonizing antibodies. This method was then used to analyse binding of trypanosomes throughout infection. In Boran cattle, the uptake of IgM coated particles manifested a steady but weak increase throughout infection, the uptake of IgG-coated parasites was not affected by the infection. In the N'Dama cattle, a strong uptake of both IgG- and IgM-coated parasites was noted two weeks after infection. As the infection progressed, the pattern of uptake resembled that in Boran cattle. These unusual kinetics resembled the kinetics of co-stimulatory cytokine release.

T2c.4 Identification of macrophage-activating factors from trypanosomes

Scientist: M. Sileghem, P. De Baetselier*, A. Darji*, D. Kennedy
Technician: R. Saya

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Immunosuppression associated with experimental trypanosomiasis is in part caused by a shift in T-cell cytokine pattern brought about by parasite-activated macrophages. When co-cultured with activated T cells, such parasite-activated macrophages increase interferon γ (IFNγ), decrease expression of interleukin 2 receptors (IL2R) and leave interleukin 2 (IL2) release unaffected. As expression of IL2R is crucial for the induction of T-cell proliferation, the cytokine shift results in a suppression of T-cell proliferation, and the whole event is usually seen as a T-cell suppression rather than a macrophage-activation. This shift in cytokine pattern was used to study the interaction of macrophages and *T. b. brucei* as a macrophage tumour hybridoma pulsed with trypanosome factors was found to cause immunosuppression as the result of a similar cytokine shift. The characterization of immunosuppression in cattle during *T. congolense* infection has revealed many similarities with *T. b. brucei* infection in mice. Therefore, the macrophage hybridoma model was used as a test system for a first screen of macrophage activating factors derived from both *T. b. brucei* and *T. congolense*.

Using ammonium sulphate precipitation, the suppressive activity was found to be present in fractions of trypanosome lysates which precipitated between 35 and 50%. The suppressive factors were further purified by ion exchange on a Mono Q column and gel filtration on a Superose 12 column. The columns were run in both FPLC and HPLC systems. In the gel filtration separations, the suppressive activity could be localized to a single fraction. Following analysis by SDS-PAGE, the fraction from *T. b. brucei* was found to contain a single protein. In the *T. congolense* fraction, four bands were detected. The fraction was further subfractionated into four subfractions of which three were negative and one was positive in the bioassay. It remains to be determined which protein is present in the positive fraction. Bioassays have also been developed in which immunosuppression can be simulated following the interaction of *T. congolense* with bovine cells. Such assays will be used to screen the different parasite fractions.

T2c.5 The effects of an anti-bovine TNF monoclonal antibody on the anaemia of cattle infected with *Trypanosoma vivax* (Galana stock)

Scientists: L. Logan-Henfrey, M. Sileghem, D. Moloo
Technicians: F. McOdimba, B. Gichuki, P. Muiya, J. Kamau, R. Saya, K. Tikolo

A mouse anti-bovine TNF-α monoclonal antibody was previously shown in an *in vitro* bioassay to neutralize the ability of recombinant bovine TNF to lyse L929 and WEHI 164 clone 13 tumour targets. The ability of this monoclonal antibody to block *in vivo* a severe onset of anaemia was assessed in Boran calves infected with the

Galana stock of *Trypanosoma vivax*. Six Boran calves 4–5 months of age were infected with *T. vivax* by allowing five infected tsetse to feed on the left flank of each animal. Three calves were randomly selected and each was given 10 mg of the monoclonal antibody intravenously once four days after infection and twice daily (20 mg) on days 9–15. The three remaining calves received intravenous injections of an equal volume of phosphate buffered saline. All the calves developed parasitaemia between days 9 and 10. The body temperature, size of the prefemoral lymph nodes draining the chancres, parasitaemias, haemograms (PCV, RBC, WBC, haemoglobin, reticulocyte counts) and bone marrow biopsies were monitored during the infection. There was no clinical difference in the progression of the anaemia or disease in the two groups of calves. One calf previously treated with the monoclonal antibody died on day 18 with a PCV of 10%. Further studies will concentrate on the ability of bovine TNF to affect bovine erythropoiesis in clonogenic assays and the blocking capacity of the bovine TNF-α monoclonal antibody in this specific system.

T2d.1 Role of ineffective haemopoiesis in the anaemia of bovine trypanosomiasis

Scientists: A. Andrianarivo, L. Logan-Henfrey, V. Anosa*, G. Gettinby†
Research Associate: C. Wells
Technicians: P. Muiya, F. McOdimba, J. Kamau

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Previous studies have suggested a role of ineffective haemopoiesis in the development of the anaemia of bovine trypanosomiasis. Using clonal assays for the committed haemopoietic progenitors, we have examined the bone marrow response during a *Trypanosoma congolense* rechallenge infection in five trypanosusceptible Boran cattle. Very early in the infection (week 2) in the absence of any detectable parasitaemia, a significant drop in the number of nucleated marrow cells was seen. A concomitant marked decrease in both the early (BFU-E) and late (CFU-E) erythroid progenitors, as well as the granulocyte-macrophage precursors (CFU-GM), was suggestive of a defect at the pluripotential stem cell level. Thereafter the BFU-E remained low throughout the infection and a return to baseline levels was only seen in one animal three weeks after treatment. Considerable individual variation was observed in the evolution of the CFU-E during the acute anaemic phase of the disease. Despite a CFU-E recovery to baseline or even higher values by 10–12 weeks after infection, the animals developed chronic anaemia with packed cell volume (PCV) levels between 17 and 22%. No erythroid progenitors have been detected in the peripheral blood prior to the challenge and during the first ten weeks of the infection. BFU-E appeared in the blood culture of two animals between weeks 11 and 14 after infection with an upsurge in one animal following trypanocidal treatment. This is the first demonstration of a negative effect on erythroid development in the bone marrow of trypanosome-infected cattle.

T2d.2 Factors regulating growth of bovine haemopoietic progenitors *in vitro*

Scientist: A. Andrianarivo
Technicians: P. Muiya, F. McOdimba

In an attempt to determine the mechanisms responsible for the altered bone marrow function in bovine trypanosomiasis [see abstract T2d.1] bone marrow plasmas from trypanosome-infected cattle were tested for the presence of possible inhibitors of haemopoiesis. Bone marrow plasmas were incorporated in the progenitors' assays and the effects on colony formation by a pool of normal bone marrow cells were assessed. Bone marrow plasma from normal donors or collected during the preinfection stage consistently induced a marked decrease in the growth of BFU-E. Heating the samples at 56 °C for 30 min resulted in partial restoration of BFU-E yield. No consistent effect on the progenitors' growth was obtained using bone marrow plasmas

from infected donors at any stage of the infection. The method was thus considered inappropriate for the detection of any inhibitory factor. Studies are in progress to examine the effects of cytokines on normal bovine bone marrow progenitors.

Several attempts have been made to improve the growth of BFU-E and more precisely the immature BFU-E in the bovine bone marrow culture system. Preliminary results showed that without any added conditioned medium the combination of bovine hemin and erythropoietin gave the best yield of both mature and immature BFU-E.

T2d.3 Enrichment of bovine haemopoietic progenitor cells by depletion of bone marrow cells with monoclonal antibodies

Scientists: J. Naessens, L. Logan-Henfrey, A. Andrianarivo
Technician: P. Muiya

Monoclonal antibodies (MAbs) are an excellent tool with which to enrich progenitor cells from the bone marrow. MAbs that recognize progenitor cells may be used to sort the cells by flow cytometry. However, it is almost impossible to screen for antibodies that detect such low percentages of cells, and only a limited number of cells can be sorted on one day. MAbs that recognize unwanted cell types may be used in combination with magnetic beads to enrich large quantities of cells in a relatively short time.

A panel of MAbs was composed from (1) those presented to the second international workshop on bovine/ovine leukocyte antigens, (2) new fusions against bovine bone marrow cells and (3) previously described MAbs that detected bone marrow cells. First, the MAbs were used to sort positive and negative cells by flow cytometry. These cell populations were identified morphologically and tested for the presence of progenitor cells in a short-term clonogenic culture assay. Most MAbs either depleted all or none of the progenitors (CFU-GM, CFU-E, late BFU-E). Only the antibody against the transferrin receptor depleted erythroid but not the myeloid progenitor cells. A number of MAbs that depleted or enriched erythroid progenitor cells could be successfully used with magnetic beads.

T2d.4 Development of a CFU-S assay for bovine haemopoietic cells in sublethally irradiated *SCID/BEIGE* mice

Scientists: L. Logan-Henfrey, J. Naessens, S. Kemp
Technicians: P. Muiya, J. Kamau, M. Opollo
Research Fellow: M. McIsaac*

*Ontario Veterinary College, University of Guelph, Canada

The generation of chimaeric bovine/immuno-deficient mice is being investigated using immune-deficient *scid/scid.bg/bg* mice infused intravenously with bovine bone marrow cells. Sublethally irradiated (4.5 Gy) *scid/bg* mice infused intravenously with

$1–2 \times 10^5$ bovine bone marrow cells developed visibly detectable foci (nodules) of haematopoietic cellular proliferation within their spleens by day 12 after inoculation. Irradiated but non-reconstituted *scid/bg* mice survived but did not develop nodules. Histologically these haematopoietic foci were located in the red pulp of the spleens near splenic arteries. The predominant cell type in foci was erythroid, consisting of many proerythroblasts and erythroblasts. The mitotic index was high. A few foci contained haematopoietic precursors of more than one cell lineage: erythroid, megakaryocytes and neutrophilic (myelocytes, metamyelocytes, bands). The late development of these nodules (12 days) and the presence of more than one cell lineage within the foci suggest that these cells developed from bovine multilineage progenitor cells and thus represent a stem cell of earlier origin than can be currently cultured *in vitro*. Monoclonal antibodies recognizing bovine leukocyte and myeloid cell antigens are being used to further characterize the cells within spleen nodules. DNA was prepared from spleens and thymus from *scid/bg* mice and was screened with bovine specific microsatellite markers by polymerase chain reaction (PCR) to ascertain that the nodules are of bovine origin. The bovine CFU-S assay may serve as a useful tool in studies of bovine infectious diseases that affect the haematopoietic system.

T2d.5 Generation of bovine cytokine reagents for studies of immunopathology

Scientists: B. Mertens, J. Naessens, L. Logan-Henfrey
Research Associate: L. Gaidulis
Technician: K. Tikolo

Haemopoietic development is controlled by a complex network of cytokines. To study the role of these factors in the anaemia of bovine trypanosomiasis, cytokine reagents have been obtained through collaborative agreements with other institutions or have been developed at ILRAD. The cDNA probes coding for bo IL-4 (courtesy of Dr. D. Dobbelaere), for bo IL-6 and bo IL-7 (both courtesy of Dr. L. Droogmans and Prof. A. Burny) and bo γ-IFN (courtesy of Dr. M. Carrington) were subcloned at ILRAD into vectors containing promoters for *in vitro* transcription. Subcloning of the cytokine c-DNA into vectors with two RNA-polymerase promoters allows their use as riboprobes. Similarly, bo TNF-α cDNA has been cloned at ILRAD. Together with reagents previously obtained under a collaborative agreement with Immunex, the list of bovine specific cytokine DNA and riboprobes held at ILRAD is as follows: bo IL-1 α, bo IL-1 β, bo IL-2, bo IL-4, bo IL-6, bo IL-7, bo-TNF-α, bo γ-IFN, and bo GM-CSF. These probes will enable the study of cytokine specific gene expression by both Northern blot analysis and by *in situ* hybridization. For each cytokine a set of primers has been synthesized and purified. These primers will be used for the amplification of cytokine specific sequences by reverse transcription polymerase chain reaction (RT-PCR) in order to obtain a relative quantitation of cytokine steady-state mRNA levels in various types of cells or tissues.

Recombinant cytokines are needed to develop bovine haemopoietic stem cell assays. Bovine IL-4 has been expressed as a fusion protein in *E. coli*. IL-4 is known for its effects on B cell differentiation although, recently, it was shown in other animals species to have effects on the formation of erythroid colonies. Both GM-CSF

and IL-3 are important growth factors in haemopoietic stem cell development, but are reportedly species specific (i.e. human and mouse GM-CSF and IL-3 do not cross react). Recombinant bo-GM-CSF and bo IL-3 are not commercially available. Recently, however, through a cooperative agreement with the Moredun Research Institute, recombinant sheep IL-3 and recombinant sheep GM-CSF were obtained. Ov GM-CSF and ov IL-3 will be compared in bovine and ovine clonogenic assays to determine if together or separately these ovine cytokines have any stimulatory effect on bovine erythroid cells.

T2d.6 Cytokine and growth factor regulation of haemopoiesis: TNF

Research Associate: L. Gaidulis
Scientists: B. Mertens, L. Logan-Henfrey, M. Sileghem

To study TNF-α gene expression in trypanosome-infected cattle, a TNF-α gene specific cDNA probe has been isolated, cloned and sequenced [see abstract T2c.1]. The polymerase chain reaction (PCR) was utilized to clone the 574 bp bovine TNF-α cDNA coding region as well as to generate a shorter 344 bp TNF-α riboprobe for *in situ* hybridization studies. Single stranded TNF-α cDNA, prepared from total RNA of lipopolysaccharide (LPS)-stimulated monocytes and bovine alveolar macrophages was used as a template in a PCR reaction with primers whose sequences were based on the conserved nucleotide segments of known mammalian TNFs. A 344 bp product was consistently amplified from the TNF induced macrophages, subcloned into a double-promoter vector, sequenced and shown to have 80–90% homology to other mammalian TNF-α genes.

The subcloned 344 bp fragment was tested for its ability to detect TNF-α-specific mRNA by Northern and Slot blot analyses. A positive signal was obtained only in total RNA from LPS-stimulated macrophages. However, using the PCR and TNF-α specific primers, mRNA could also be detected in ss cDNA from unstimulated alveolar macrophages, demonstrating the increased sensitivity of the PCR as a tool for detecting cytokine mRNA levels.

Southern blot analysis of restriction digests of bovine genomic DNA showed that the 344 bp probe detected a single allele in both N'Dama and Boran cattle with *EcoR*I, *Xba*I, *BamH*I, *Sau*III A and *Taq*I digests. However, with *Hind*III digests, two alleles were detected in the Boran breed. This polymorphism is being further investigated.

The full length 574 bp TNF-α cDNA coding region of both the N'Dama and Boran cattle has been cloned and sequenced. Sequencing differences between the two breeds will be further studied to determine if they represent PCR errors among different ss cDNA populations of mRNA used in the PCR cloning of bovine TNF-α.

Work is currently in progress to express bovine TNF-α in *E. coli* in order to produce bulk quantities of recombinant protein for use in clonogenic methyl cellulose progenitor cell assays.

T2d.7 Erythropoietin responses in *Trypanosoma congolense*-infected cattle

Research Fellow: J. Buza
Supervisors: L. Logan-Henfrey, D. Williams

The anaemia of *Trypanosoma congolense* infections in cattle is associated with a poor erythropoietic response. Erythropoietin is a glycoprotein hormone produced primarily in the kidney in response to hypoxia and is the principal regulator of erythropoiesis. Inadequate levels of erythropoietin in the plasma of trypanosome-infected cattle has been suggested as a possible cause of the inadequate erythropoiesis.

Two assays, which are used to measure erythropoietin in human plasma, were assessed as to their ability to measure bovine erythropoietin in the plasma of calves made anaemic either by sudden blood loss of 50 % of their estimated total blood volume or by a natural *T. congolense* infection. Haematological changes were examined in both groups of calves.

As bovine erythropoietin is not commercially available, a radioimmunoassay was developed using the human urinary erythropoietin as the standard, the anti-human urinary erythropoietin polyclonal antisera as a binder and recombinant human erythropoietin labelled with I^{125} as the tracer. The assay could detect erythropoietin in human serum at a minimum detection limit of 10 mU/ml. However, the radioimmunoassay failed to detect erythropoietin in serum from an anaemic calf, suggesting that this particular polyclonal antibody to human erythropoietin did not recognize bovine erythropoietin in this immunological assay.

A bioassay which indirectly measures erythropoietin levels *in vitro* by utilizing erythropoietin's stimulatory effect on I^{125}-deoxyuridine (IUDR) incorporation to DNA of spleen cells from phenylhydrazine treated mice was able to measure erythropoietin levels in bovine plasma. The assay could detect erythropoietin in plasma at a minimum detection limit of 62.5 mU/ml. Using the bioassay, increased erythropoietin levels were detected in calves as early as six hours post-bleeding (hpb). The peak levels of 1225 mU/ml was reached at 33 hpb and dropped below the detection limit of the assay by 72 hpb. Reticulocytes appeared in the blood of the calves 72 hpb and this was followed by a macrocytic hypochromic anaemia during the recovery period. From 18 days after infection, undiluted plasma collected from *T. congolense*-infected animals suppressed IUDR incorporation into spleen cells. Diluting the plasma five-fold decreased its suppressive effect and allowed the detection of erythropoietin. Using diluted plasma in the assay, distinct erythropoietin peaks were observed both in the acute stage of the disease when the packed cell volume (PCV) was dropping rapidly and in the chronic stage when the PCV had stabilized between 16–17%. Increased erythropoietic response occurred throughout the acute stage of the disease indicated by progressive increase in mean corpuscular volume. During the chronic stage of the disease (39–76 days after infection) the anaemia was macrocytic hypochromic and there were very few reticulocytes (0.2–0.4%). From 76 days following infection, despite low PCV and peaks of elevated erythropoietin, the erythropoietic response waned as indicated by a normocytic normochromic anaemia and an absence of reticulocytes. This suggests the bone marrow was unresponsive to elevated plasma erythropoietin. Cattle treated in the chronic stage of *T. congolense* infections recovered their normal PCV although their erythropoietin levels were

below the detection limit of the assay. The animal treated on day 57 of infection recovered its pre-infection PCV four weeks after treatment while an animal treated on day 85 had only recovered 84% of its pre-infection PCV eleven weeks after treatment.

T2e.1 Bovine genetic markers—microsatellite identification and analysis

Scientists: L. Brezinsky, S. Kemp
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In a search for bovine polymorphic microsatellite sequences, 99 clones of genomic DNA containing the microsatellite (dG-dT)n.(dC-dA)n from male N'Dama *(Bos taurus)* subgenomic libraries have been isolated and sequenced. On the basis of 46 of these sequences, pairs of primers for the polymerase chain reaction (PCR) were designed. Forty pairs have been screened for usefulness in detecting polymorphism in N'Dama and Boran cattle. Fifteen have yielded informative microsatellite markers. The 25 unsuccessful primer pairs were rejected for one of two reasons—four yielded signals with N'Dama but not Boran template DNA and the remaining 21 failed to give a specific (single locus) PCR product, in most cases due to their association with repetitive elements. The segregation of 11 of the 15 characterized microsatellite markers has been studied in two large N'Dama × Boran families (ND8 × 1688 and ND7 × 1419 with 36 and 29 calves respectively). There were no inconsistencies in the pattern of inheritance indicating that there are no parentage errors in the families and that spontaneous mutation of microsatellite sequences is unlikely to limit their usefulness.

The same 11 markers were also screened against a panel of DNAs prepared from goat, buffalo, sheep and a number of wild ruminants, together with samples from horse and pig. Different subsets of the marker panel were found to be informative in different species. Seven of the markers were informative in both sheep and goats.

T2e.2 Bovine genetic markers—polymorphic markers in specific genes

Research Fellow: M. Agaba
Scientist: S. Kemp

The 3′ untranslated (3′UT) regions of sequenced genes are a potential source of polymorphic markers to characterize trypanotolerant N'Dama and susceptible Boran cattle. Although simple sequence variations in such regions provide less polymorphism than microsatellites, they have the advantage of being associated with parts of the genome which are translated and which in many cases are assigned to chromosomes.

Oligonucleotide primers defining the 3′UT regions of selected genes have been designed and used to initiate polymerase chain reaction (PCR) amplification of the regions in DNA of a Boran cow and an N'Dama bull. The amplification products from each animal were directly sequenced. Sequence differences between the two animals were then used as the basis for designing PCR primers giving allele-specific amplification.

The value of the approach was initially demonstrated with the gene for bovine gamma-crystallin, a member of an important syntenic group. Bovine parathyroid

hormone (PTHG) and prolactin hormone (PRLP) loci have now also been analysed. Approximately 340 bp of the 3'UT region of N'Dama and Boran PRLP were sequenced but no polymorphism was found. In the case of PTHG, a T to C transition was identified at position 2430; the N'Dama carried a C at this position (as in the published sequence) and the Boran carried a T. Primers were designed which, together with one of the primers originally used to amplify the 3' UT region, enabled specific detection of either of the alleles. This has been applied to samples obtained from an N'Dama/Boran cross-bred herd. The 'N'Dama-type' allele is homozygous in all of the N'Dama founders of the herd and the 'Boran-type' allele is homozygous in the Boran founders. Thus this locus will be fully informative in the F2 generation.

T2e.3 Bovine genetic markers—randomly amplified polymorphic DNA

Scientists: S. Kemp, A. Teale

In an attempt to rapidly identify population-specific DNA markers for use in cattle, two pools of genomic DNA were made comprising equal proportions of samples from 10 N'Dama animals *(Bos taurus* of Gambian origin) and 10 Boran animals *(B. indicus)*, respectively. Each of the breed pools was amplified by the polymerase chain reaction (PCR) with 80 10 bp random sequence oligonucleotide primers used singly. Four of the primers generated pool-specific products visible in ethidium bromide-stained agarose gels. Each of the four primers was then used to amplify the individual DNA components of the pools. One of the primers (ILo 526) revealed the pool-specific product in all of the Boran DNA amplifications but not in any of the N'Dama amplifications.

The ILo 526 primer was then used to amplify the DNA of a number of representative *Bos indicus* individuals of the Brahman and East African Zebu (Maasai) breeds. The 'Boran' product was generated in all cases. The product was not generated in amplifications of any Friesian animals or of N'Dama animals of Gabon origin. The product was observed in amplifications of some animals of the Creole breed, but not all. (The Creole breed is considered to be of mixed *B. indicus* and *B. taurus* origin.)

Using the pooled DNA approach with random primed PCR, it has been possible to rapidly identify a bovine subspecies-specific DNA marker. The nature of the marker DNA and its sequence variations are currently under investigation.

T2e.4 Reference and resource populations of cattle for the analysis of trypanotolerance

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

For the purposes of linkage analysis of genetic markers and genes controlling the trypanotolerance trait, the objective has been to produce four full-sibling F1 families of N'Dama × Boran cattle from which to breed an F2 generation segregating trypanotolerance. Two of the F1 families (sire/dam ND7/1419 and ND8/1688) had

been completed before the end of 1991 with 29 and 36 animals born. At the end of 1992, the remaining two F1 families, ND9/1801 and ND10/2094, comprised 11 and 9 calves respectively, with five calves *in utero* in both cases.

The design of the F2 generation has been reconsidered during the past year in the light of the requirements for application of randomly amplified polymorphic DNA (RAPD) markers and interpretation of RAPD genotypes [see abstract T2e.3]. A smaller number (4–8) of larger full-sibling F2 families (40–20 members) than originally planned will be made in order to facilitate use of this marker class. This will not devalue the population with respect to use of other marker types.

Construction of the 160-member F2 generation has begun with breeding between the ND7/1419 and ND8/1688 families. To date, a total of 16 F2 animals are *in utero* and 25 other embryos have been implanted (pregnancies in these latter cases remain to be confirmed). The first F2 calves were born in November 1992.

At the XXIII International Conference on Animal Genetics, it was agreed that the mapping community would employ developing markers on a common set of families for the purpose of development of a consensus map with useful marker density. These will include ILRAD's ND7/1419 and ND8/1688 families.

SOCIOECONOMICS

SEP1 Production, delivery and adoption of alternative improved control measures for livestock diseases

Scientists: J. Curry, A. Mukhebi, B. Perry
Collaborating Scientists: T. Dolan, P. Lessard, S. Morzaria, A. Teale, A. Young, G. Mullins*, W. Thorpe*, B. Swallow*, collaborating scientists from the Kenya Agricultural Research Institute (KARI) and the Kenya Trypanosomiasis Research Institute (KETRI)
Research Associate: R. Kruska

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This project is currently assessing the economic implications of successful delivery and adoption of the infection-and-treatment method of immunization for East Coast fever (ECF) on a case-study basis, from which it is hoped to draw broader conclusions relating to the successful application of this technology over wider areas of eastern, central and southern Africa.

In a study in coastal Kenya, work was carried out in collaboration with the Kenya Agricultural Research Institute (KARI) and the International Livestock Centre for Africa (ILCA), and costs of delivering pilot immunization were calculated. Immunization against ECF at a calculated pilot cost to the farmer of Kshs. 544 (US$ 25) per animal in Kaloleni Division was calculated to be financially profitable in grade cattle of the region, with benefit:cost ratios ranging from 2:1–5:1. However, the benefits from immunization at this cost would not be sufficient to justify immunization of Zebu cattle, the dominant cattle type in much of coastal Kenya. For these animals, the cost of immunization to the farmer would have to be lower, in the range of Kshs. 230 to 415 per animal, or the farm–gate price of milk would have to be increased by at least 80% from the current price of Kshs. 7.50 to 13.50 per litre, or the government would have to subsidize the delivery cost, either partially or fully. The first two prospects are realistic, as cost of routine immunization are likely to be lower then those incurred at the pilot level, and as the increasing demand for milk is likely to inflate prices in the liberalized markets. Issues of vaccine pricing and subsidy to the farmer in regard to ECF immunization centre on equity, and involve policy decisions generally in the hands of the government.

In collaboration with ILCA and the Kenya Trypanosomiasis Research Institute (KETRI), a study to investigate the socioeconomic factors affecting the adoption and sustainability of community-based tsetse control programs utilizing a range of control technologies has been initiated. The objectives of this research are: a) to determine the extent of community knowledge of trypanosomiasis and its vectors, and of the socioeconomic importance attached to the disease by individuals and communities; b) to identify the relevant socioeconomic factors which influence individuals and communities to support community-based tsetse control programs; c) to understand the relative importance of these factors in forming attitudes towards support and participation in such projects; d) to assess the affordability and economic sustainability of community-based tsetse control programs; and e) to develop efficient *ex ante* methodologies to assess levels for community support that can be used for similar community-based programs. By seeking to determine the extent of community

knowledge about trypanosomiasis and its vectors and to identify factors likely to affect adoption of different control technologies by individuals and communities, this project hopes to identify factors at the national and farm levels which are essential for the successful production, delivery and adoption of alternative improved control measures for livestock diseases.

Preliminary information obtained so far from field interviews suggests that variation in knowledge of the disease is dependent upon the degree of contact the community has had with external sources of information and disease control. A provisional typology of communities based on their disease knowledge, ranging from no knowledge at all to knowledge based on long-term familiarity with the disease, has been constructed.

SEP2 Epidemiological aspects relating to the application of improved disease control measures for tick-borne diseases

Scientists: B. Perry, A. Mukhebi, J. Curry
Research Associates: R. Kruska, J. Katende
Visiting Research Fellows: S. Deem*, C. O'Callaghan†
Collaborating Scientists: T. Dolan, J. McDermott†, S. Morzaria, A. Musoke, A. Young, J. Rowlands‡, W. Thorpe‡, G. Medley§, D. Rogers¶, S. Randolph¶, collaborating scientists from national agricultural research services in Kenya and Zimbabwe

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Assessing the economic importance of diseases and the impact of disease control relies on accurate data on current distribution and occurrence of diseases, and on their effect under different conditions of livestock production economics. With the scarcity of such data with regard to tick-borne diseases on the African continent, the program has explored several modelling approaches to predict their distribution and occurrence, and some of these have been reported in previous years. Further development has been made of models to predict (tick) vector and disease (theileriosis) distribution using discriminant analysis in a geographical information system (GIS), developed in collaboration with the University of Oxford. A model developed in collaboration with Imperial College, London to predict the dynamics of theileriosis under specific conditions (for which adequate data were available to validate the model) has been completed. Based on a differential equation methodology, this model accurately predicts the relationships between antibody prevalence in a cattle population, the incidence of theileriosis and case-fatality.

One of the most valuable precursors for the establishment of disease impact data is serological prevalence rates, if assessed in the right age group of cattle with the right serological test. However, also important is the correct study design and animal

sampling procedure if prevalence rates are to be valid and truly represent the situation in the cattle population from which they were taken. In collaboration with KARI and ILCA, a study has been conducted to investigate statistical procedures required to determine serological prevalence rates of tick-borne diseases in different agroecological zones. Under the conditions of the study, carried out in Zebu cattle in Coast Province, Kenya, and taking into consideration the interrelationships between endemic stability of tick-borne diseases, prevalence rates, intraherd correlations and acceptable error, it was suggested that a sample size of 10–20 herds that contain an average of 10 calves per herd may be adequate across a range of different epidemiological states, with sample sizes at the top end of the range required in endemically unstable areas. In larger herds, random subsampling of calves within herds would be acceptable and help to reduce costs. These sampling considerations should be taken into account in further studies to determine the associations between antibody prevalence rates, disease incidence rates and case-fatality with a view to determining appropriate control strategies for East Coast fever.

SEP3 Socioeconomic and environmental impact of the application of improved disease control measures for livestock diseases

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Research Associate: R. Kruska
Collaborating Scientists: T. Dolan, S. Leak, P. Lessard, S. Morzaria, A. Teale, A. Young, P. de Leeuw*, G. Mullins*, J. Rowlands*, B. Swallow*, W. Thorpe*, J. Ellis†, collaborating scientists from national agricultural research services in Kenya and Zimbabwe

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Two computer spreadsheet models are being developed by ILRAD, with consultancy input from A.P. Consultants of the UK, for assessing economic losses due to theileriosis and trypanosomiasis in cattle in Africa, and the economics of alternative control strategies under different livestock production systems. One model provides analysis at site/country level while another model provides an aggregated analysis of several countries affected by the disease. The models have been tested using data from eastern and southern Africa for theileriosis (reported previously), and this year has seen further application of the model to Coast Province, Kenya, and the start of an application to Zimbabwe. At the Kenya Coast, the model predicts that East Coast fever (ECF) immunization would reduce economic losses by 24–40% in indigenous Zebu cattle, and by 40–70% in genetically improved grade cattle, yielding increases in net income of 24–103% under a variety of alternative control scenarios. The benefit:cost ratio from immunization ranged from 2–5. This model is being applied for similar analyses of theileriosis in Zimbabwe and Uganda, and will also be applied to assess the economic impact of heartwater control, with an initial study in Zimbabwe in collaboration with the Department of Veterinary Services and the University of Florida Heartwater Project.

Preliminary results of the two models on the economics of trypanosomiasis for two countries (Gambia and Zimbabwe) were very similar. For instance, the site/country model estimated the annual losses in the Gambia and Zimbabwe to be US$ 400,000 and US$ 6.2 million respectively. Modifications are being made to both models to reconcile differences in results. Further analysis and testing of these models are being undertaken with data from three other countries, with a view to developing an initial estimation of the continent-wide losses due to trypanosomiasis.

SEP4 Application of the Technology Impact Evaluation Simulator model to determine the impact of disease control measures in two localities in Kenya

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Scientists: A. Mukhebi, J. Curry, B. Perry

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A whole farm-level simulation model, the Technology Impact Evaluation Simulator (TIES), was developed at Texas A & M University, USA, and ILRAD and was described in previous reports. The model has been used to simulate the economic impact of immunizing grade and Zebu cattle against ECF by the infection-and-treatment method under alternative acaricide application strategies in two contrasting livestock production systems in Kenya: the relatively large-scale farms in Uasin Gishu District in the Kenyan highlands and the relatively small-scale farms in Kaloleni Division in the Kenya coastal lands. The alternative control strategies involved reduction in acaricide application intensity from zero up to 75% following immunization.

The financial performance of farms and the probability of their economic survival under the alternative control strategies were evaluated. The strategies were also evaluated for farmer-response to agricultural risk, using the criterion of stochastic dominance with respect to a function, and determining the most preferred strategy by farmers with an estimate of the associated confidence premiums.

The results indicated that immunization was financially superior to the current acaricide application control method under all control scenarios. The most preferred ECF control strategy was the adoption of immunization with a 75 percent reduction in acaricide use. In general, Uasin Gishu farms, which kept predominantly grade cattle, would generate greater returns from adopting immunization compared to Kaloleni farms, which kept predominantly Zebu cattle. In both areas, grade cattle would generate greater returns from ECF immunization than Zebu cattle.

The estimated confidence premiums associated with the most preferred control strategy were Kshs. 1,285 and 4,379 for Zebu and grade cattle, respectively, for Kaloleni Division. This implies that grade cattle farmers face a higher risk from ECF than Zebu cattle farmers, and that Kaloleni farmers face a higher risk of the disease than those in Uasin Gishu. The TIES model offers a flexible method for financial analysis of new technologies incorporating risk on smallholder farms. It can be applied for farm level analysis of alternative control strategies for other livestock diseases.

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STAFF PRESENTATIONS AT INTERNATIONAL MEETINGS

A. Andrianarivo

Keystone Symposium in Molecular and Cellular Biology, Tamaron, Colorado, USA, February, 1992. Paper presented: *Bone marrow response to a* Trypanosoma congolense *rechallenge infection in Boran cattle.* A. Andrianarivo, L. Logan-Henfrey, P. Muiya and F. McOdimba.

R. Bishop

Workshop on Genome Analysis of Protozoan Parasites, ILRAD, Nairobi, November, 1992. Paper presented: *Genomic polymorphisms in* Theileria parva. R. Bishop, H. Baylis, B. Allsopp, P. Toye, V. Nene, T. Dolan, P. Spooner, M. Kibe and S. Morzaria.

J. Curry

International Livestock Seminar on Livestock Services for Small-Holder Farmers, Yogyakarta, Indonesia, November, 1992. Papers presented:

1. *Improving animal health care through the use of ethnoveterinary knowledge: examples for coastal and highland Kenya.* J. Curry, B. Berry, J. Delehanty and S. Mining.
2. *Assessing the impact of livestock disease control using dietary and nutritional measures in two sites in rural Kenya.* R. Huss-Ashmore, G. Mullins and J. Curry.
3. *Targeting small holder livestock against East Coast fever in Uasin Gishu District, Kenya.* A. Mukhebi, J. Curry, B. Perry, S. Mining, J. Delehanty, R. Huss-Ashmore and R. Kruska.

Meeting on Operational Research on African Trypanosomiasis, World Health Organization/Uganda Trypanosomiasis Research Organisation, Tororo, Uganda, September, 1992. Paper presented: *Evaluation methodologies in the social sciences.*

91st Annual Meeting of the American Anthropological Association, San Francisco, California, USA, December, 1992. Paper presented: *Gender and livestock disease control in Uasin Gishu District, Kenya.* J. Curry, R. Huss-Ashmore, A. Mukhebi and B. Perry.

Seminar on Integrated Approach to Livestock Improvement with Special Reference to East Coast fever Immunization in Kilifi District, Coast Province of Kenya, Mombasa, Kenya, November, 1992. Paper presented: *Livestock in household economy and the economics of East Coast fever immunization in Kaloleni Division, Kenya.* A. Mukhebi, E. Mussukuya, D. Kariuki, P. Ngumi, B. Perry, W. Thorpe, J. Curry and G. Mullins.

C. Daubenberger

European Conference on Basic and Clinical Aspects of Macrophage Biology, Regensburg, Germany, September, 1992.

M. Dirie

1st International Seminar on Non-Tsetse Transmitted Trypanosomiasis, Annecy, France, October 1992. Paper presented: Trypanosoma vivax *isolates from Colombia.*

T. Dolan

Future of Livestock Industries in East and Southern Africa, Kadoma, Zimbabwe, July, 1992. Papers presented:

1. *Sustainable methods for tick and tick-borne diseases in Africa.* A. Latif and T. Dolan.
2. *Immunization of cattle against East Coast fever on Zanzibar.* K. Biwi and T. Dolan.

3rd International Veterinary Immunology Symposium. Budapest, Hungary, August, 1992.

J. Doyle

7th International Conference of Institutions of Tropical Veterinary Medicine, Yamoussoukro, Côte d'Ivoire, September, 1992. Paper presented: *What kind of animal health strategies for different agricultural production systems?*

W. Fish

Keystone Symposium, Taos, New Mexico, March, 1992.

P. Gardiner

1st International Seminar on Non-Tsetse Transmitted Trypanosomiasis, Annecy, France, October 1992. Paper presented: Trypanosoma vivax *in Africa and South America: our state of knowledge compared.*

International Livestock Seminar on Livestock Services for Small-Holder Farmers, Yogyakarta, Indonesia, November 1992. Paper presented: *The International Laboratory for Research on Animals Diseases: Relating modern science to the needs of the livestock farmer.*

H. Hirumi

45th Annual Meeting of the Society of Protozoologists, Vancouver, Canada, July, 1992. Paper presented: *An* in vitro *method for detecting sensitivity of* Trypanosoma congolense *bloodstream forms to four trypanocidal drus* and a video film on *'Complete cyclic development of* Trypanosoma vivax in vitro'.

M. Juarrero

New Generation Vaccines, The Role of Basic Immunology, Cape Soumon Beach, Greece, July, 1992. Paper presented: *Recombinant viruses expressing p67 antigen from* Theileria parva *sporozoites.*

J. Katende

6th International Symposium of the World Association of Veterinary Laboratory Diagnosticians, Lyon, France, June 1992. Paper presented: *Antibody and antigen capture ELISAs based on a 200-kDa antigen of* Babesia bigemina.

S. Kemp

23rd International Conference on Animal Genetics, Interlaken, Switzerland, August, 1992. Poster presented: *Random amplified DNA polymorphisms (RAPDs) and pooled DNA in bovine genetic studies.* S. Kemp and A. Teale.

Conference on the 'Human Genome 92', Nice, France, October, 1992.

L. Logan-Henfrey

The SCID Mouse in Biomedical and Agricultural Research, University of Guelph, Ontario, Canada, August, 1992. Paper presented: *Development of a CFU-S assay for bovine haematopoietic cells in sublethally irradiated SCID/BIEGE mice.* L. Logan-Henfrey, L. Muiya and P. Naessens.

21st Annual Meeting of the International Society for Experimental Haematology, Providence, Rhode Island, USA, July, 1992.

Workshop on Genome Analysis of Protozoan Parasites, ILRAD, Nairobi, November, 1992. Paper presented: *Animal Trypanosomiasis: The International Laboratory for Research on Animal Diseases meets the challenge.*

N. MacHugh

3rd International Veterinary Immunology Symposium, Budapest, Hungary, August, 1992. Paper presented: *A monoclonal antibody specific for bovine CD45RO differentiates memory from naive CD4 positive bovine T cells.* N. MacHugh, E. Awino and D. McKeever.

P. Majiwa

An International ICRO/UNESCO Training Course on the Biochemistry of Parasitic Protozoa, jointly organized by the Department of Biochemistry, University of Nairobi, and the International Laboratory for Research on Animal Diseases, August 1992. Course lecturer.

UNDP/EEC International Group Training Course on Integrated Tsetse Management for the Tropical Developing World, November, 1992. Paper presented: *Trypanosome diagnosis and characterization using recombinant DNA.*

R. Masake

6th International Symposium of the World Association of Veterinary Laboratory Diagnosticians, Lyon, France, June 1992.

An International ICRO/UNESCO Training Course on the Biochemistry of Parasitic Protozoa, jointly organized by the Department of Biochemistry, University of Nairobi, and the International Laboratory for Research on Animal Diseases, August 1992. Paper presented: *The use of antigens for identification of protozoan parasites.*

D. McKeever

3rd International Veterinary Immunology Symposium, Budapest, Hungary, August, 1992. Paper presented: *The role of class I MHC-restricted cytotoxic T cells in protection of immune cattle from* Theileria parva.

Keystone Symposium, Taos, New Mexico, March, 1992. Paper presented: *Antigen presentation function of the MHC.*

S. Morzaria

5th International Congress of Infectious Diseases, Nairobi Kenya, June, 1992. Poster presented: *New methods in the diagnosis of* Babesia. S. Morzaria, J. Katende, A. Kairo, V. Nene and A. Musoke.

1st International Conference on Tick-Borne Pathogens at the Host-Vector Interface: an Agenda for Research, St. Paul, Minnesota, USA, September, 1992. Papers presented:

1. *Evidence of a sexual cycle in* Theileria parva *and characterization of the recombinants.* S. Morzaria, J. Young, P. Spooner, T. Dolan, A. Young and R. Bishop.
2. *The ecology of* Theileria parva *infections of cattle and the development of endemic stability.* B. Perry, S. Deem, G. Medley, S. Morzaria and J. Young.
3. *Factors affecting the transmission of African* Theileria *species of cattle by ixodid ticks.* A. Young, M. Shaw, H. Ochanda, S. Morzaria and T. Dolan.

3rd Symposium on Tropical Animal Health and Production: Bovine Theileriosis, Utrecht, Holland, October, 1992. Paper presented: *The Biology of* Theileria parva *and its importance in the development of a novel vaccine.* S. Morzaria, A. Musoke, J. Young and V. Nene.

A. Mukhebi

ILRAD/FAO Workshop on the modelling of Vector-Borne Diseases and Other Parasitic Diseases, ILRAD, Nairobi, Kenya, November, 1992. Paper presented: *Needs for modelling socioeconomic and environmental impacts of livestock disease control.*

Meeting of the CGIAR Social Scientists, ISNAR, The Hague, The Netherlands, August, 1992. Paper presented: *Issues in field data collection and modelling for assessing impacts of improved livestock disease control.* A. Mukhebi, J. Curry and B. Perry.

A. Musoke

1st African Immunology Meeting, Harare, Zimbabwe, February, 1992. Paper presented: *Immunization against* Theileria parva.

Technical Advisory Group Meeting, USAID Cooperative Agreement, Gainesville, Florida, May, 1992. Paper presented: *Improved animal vaccines through biotechnology: Phase II, anaplasmosis and babesiosis.*

8th International Congress of Immunology, Budapest, Hungary, August 1992.

Symposium on Recombinant and Synthetic Vaccines, New Delhi, India, December, 1992. Paper presented: *A recombinant vaccine of* Theileria parva.

J. Naessens

8th International Congress of Immunology, Budapest, Hungary, August 1992. Paper presented: *Resting bovine B cells do not have detectable surface IgD.*

3rd International Veterinary Immunology Symposium, Budapest, Hungary, August, 1992. Paper presented: *Bovine CFU-S in irradiated SCID/BIEGE mice.* J. Naessens, P. Muiya and L. Logan-Henfrey.

2nd International Bovine and Ovine Leukocyte Antigen Workshop, Budapest, Hungary, August, 1992. Papers presented:

1. *Biochemical characterization of three bovine non-lineage antigens by workshop antibodies (BoCD44, BoWC9 and BoWC11).* J. Naessens, and J. Nthale.
2. *Cross-reactivity of second workshop antibodies and ruminant cells.* J. Naessens, R. Olubayo, W. Davis and J. Hopkins.
3. *Expression of antigens on haemopoietic precursor cells in bovine bone marrow.* P. Muiya, L. Logan-Henfrey and J. Naessens.

D. Nandan

International Symposium on Recombinant and Synthetic Vaccines, New Delhi, India, December, 1992.

V. Nene

Keystone Symposium on Molecular and Cellular Biology of Host-Parasite Interactions, Park City, Utah, USA, January, 1992. Paper presented: *A subset of* Theileria parva *mitochondrial proteins is encoded by a linear 7.1 kbp DNA molecule.*

An International ICRO/UNESCO Training Course on the Biochemistry of Parasitic Protozoa, jointly organized by the Department of Biochemistry, University of Nairobi, and the International Laboratory for Research on Animal Diseases, August 1992. Paper presented: *Identification of* Theileria parva *antigens.*

International Symposium on Current Research Trends on The Biochemistry of Parasitic Protozoa, Nairobi, August, 1992. Paper presented: *Towards vaccine development:* Theileria parva.

Workshop on Genome Analysis of Protozoan Parasites, ILRAD, Nairobi, November, 1992. Paper presented: Theileria parva *goals and problems.*

H. Nyangito

International Livestock Seminar on Livestock Services for Small-Holder Farmers, Yogyakarta, Indonesia, November 1992. Paper presented: *Simulation modelling for analysing impacts of improved livestock disease control technologies for small-holder farmers: the case of East Coast fever in Kenya.* H. Nyangito, J. Richardson and A. Mukhebi.

O. ole-MoiYoi

African Biotechnology Networks, Nairobi, Kenya, February, 1992. Paper presented: *Application of biotechnology for improvement of animal and human health.*

Human Genome Organization, Caxambu, Minas Gerais, Brazil, May, 1992. Paper presented: *Application of modern biological techniques to solving development problems in Africa.*

The Howard Hughes Program in the Life Sciences, Wesleyan University, Massachusetts, USA, July, 1992. Paper presented: *Molecular aspects of cellular transformation induced by intracellular protozoan parasite* Theileria parva.

Department of Biochemistry, The Swiss Institute for Cancer Research, Eppalinges/Lausanne, August, 1992. Paper presented: *Induction of casein kinase II is associated with lymphocyte transformation by* Theileria.

Annual Meeting of the American Society of Tropical Medicine and Hygiene, Seattle, Washington, USA, November, 1992. Paper presented: *Protein kinases of parasitic protozoa.*

UNESCO's Scientific Coordinating Committee—Meeting on Training in Science, UNESCO Headquarters, Paris, December, 1992.

B. Perry

Division of Viral and Rickettsial Diseases Seminar, Centre for Disease Control, Atlanta, USA, January, 1992. Paper presented: *Predicting the epidemiology of tick-borne diseases using geographical information systems.*

Nairobi Cluster Seminar on Tick-Borne Diseases, Nairobi, Kenya, January, 1992. Paper presented: *Modelling the epidemiology of tick-borne diseases.*

Veterinary Research Laboratory Seminar, Harare, Zimbabwe, February, 1992. Paper presented: *Modelling the epidemiology of theileriosis: an aid to decisions on its control.*

M. Shaw

5th International Congress on Cell Biology, Madrid, Spain, July, 1992. Papers presented:

1. *Cell biology of* Theileria*—lymphocyte interaction.*
2. *The role of calcium in* Theileria parva *sporozoite entry into bovine lymphocytes.*

6th European Multicolloquium of Parasitology, The Hague, Holland, September, 1992. Paper presented: *The secretory apparatus of* Theileria parva *schizonts*. M. Shaw, L. Tilney and R. Njamunggeh.

M. Sileghem

8th International Congress of Immunology, Budapest, Hungary, August 1992. Paper presented: *Role of prostaglandins and interferon-gamma in suppression associated with tsetse-transmitted trypanosomiasis in cattle*. M. Sileghem, J. Flynn and P. Baetseller.

A. Teale

Miami Biotechnology Winter Symposium: Feeding the World in the 21st Century, Miami, January, 1992.

23rd International Conference on Animal Genetics, Interlaken, Switzerland, August, 1992. Posters presented:

1. *PCR analysis of mitochondrial DNA polymorphism in N'Dama and Zebu cattle*. R. Suzuki, S. Kemp and A. Teale.
2. *A panel of bovine microsatellite genetic markers*. L. Brezinsky, S. Kemp and A. Teale.

M. Touré

OIE Annual Meeting, Paris, France, May, 1992.

7th International Conference of Institutions of Tropical Veterinary Medicine, Yamoussoukro, Côte d'Ivoire, September, 1992.

D. Williams

1st International Union of Immunology Societies African Immunology Meeting, Harare, Zimbabwe, February, 1992. Paper presented: *Experimental infection with a haemorrhage-causing* Trypanosoma vivax *in N'Dama and Boran cattle*. D. Williams, L. Logan-Henfrey, E. Authié, C. Seely and S. Moloo.

3rd International Veterinary Immunology Symposium. Budapest, Hungary, August, 1992. Paper presented: *Specificity of seroantibodies derived from* Trypanosoma congolense-*infected cattle*. D. Williams, K. Taylor, J. Newson and J. Naessens.

J. Wilkes

Molecular Parasitology Meeting, Woods Hole, Massachusetts, USA, September 1992. Paper presented: *Uptake of the trypanocide Samorin in populations of* Trypanosoma congolense *is related to drug sensitivity*. J. Wilkes, D. Zilberstein, H. Hirumi and A. Peregrine.

Y. Yagi

114th Japanese Conference on Veterinary Science, Sapporo, Japan, September, 1992. Paper presented: *Pathogenesis of the anaemia and alteration of erythrocyte metabolism of* Theileria sergenti.

A. Young

Nairobi Cluster Tick and Tick-Borne Disease Section Symposium, ILRAD, Nairobi, January, 1992. Paper presented: *Biological aspects of the epidemiology of theileriosis—new concepts.*

21st Congress of the Parasitology Society of Southern Africa, Parasitology into the 21st Century, Middelburg, South Africa, June, 1992. Paper presented: *Prospects for improved control of tick-borne diseases in Africa.* A. Young, S. Morzaria, T. Dolan and B. Perry.

International Congress of Infectious Diseases, Nairobi, June, 1992.

First International Conference on Tick-Borne Pathogens at the Host-Vector Interface, Saint Paul, Minnesota, USA, September, 1992. Paper presented: *Factors affecting the transmission of African* Theileria *species of cattle by ixodid ticks.* A. Young, M. Shaw, H. Ochanda, S. Morzaria and T. Dolan.

Workshop on Genome Analysis of Protozoan Parasites, ILRAD, November, 1992.

Workshop on the Modelling of Vector-Borne and Other Parasitic Diseases, ILRAD, November, 1992. Papers presented:

1. *Tick-vector population dynamics: ILRAD's requirements.*
2. *Transmission of* Theileria: *ILRAD's requirements.*

SHORT-TERM VISITING SCIENTISTS

Name	Project	Dates
Mr. A. Boulangé IEMVT France	Trypanosome protein antigen sequencing	11/2/91—
Dr. J. Ellis Natural Resources Ecology Laboratory Colorado State University USA	Studies of environmental impact of trypanosomiasis control	13/1/92–24/1/92 15/9/92–19/12/92
Prof. R.O. Arowolo University of Ibadan Nigeria	Chemotherapy of trypanosomiasis	20/1/92–27/7/92
Dr. A. Shaw AP Consultants England	Economic impact of trypanosomiasis	20/1/92–22/1/92
Dr. D. Zilberstein Israel Institute of Technology Haifa, Israel	Isometamidium uptake by trypanosomiases	28/1/92–18/2/92 7/7/92–5/9/92
Prof. L.G. Tilney Department of Biology University of Pennsylvania USA	Biology of *Theileria parva*	1/2/92–15/3/92
Dr. M. Kodama Ministry of Agriculture, Forestry and Fisheries Tsukuba, Japan	Bovine monoclonals	4/2/92–26/4/92
Ms. A. Sheriff Strathclyde University Scotland, UK	Tick database analysis	24/2/92–5/4/92
Dr. D. Berkvens Belgian Animal Disease Control Project, Chipata Zambia and/or Prinz Leopold Institute, Belgium	ECFXpert computer program/Epidemiological modelling for tick-borne diseases	1/3/92–15/3/92 2/8/92–16/8/92
Prof. G. Gettinby Strathclyde University Scotland, UK	Consultant statistician Disease and herd production models	1/3/92–15/3/92 15/9/92–28/9/92

Name	Project	Dates
Dr. T. Baltz University of Bordeaux France	Consultations on trypanosomiasis research	15/2/92–29/2/92
Dr. G.C. Russel IAPGR/AFRC Scotland, UK	PCR amplification of Class II molecules	15/2/92–14/3/92
Dr. W. I. Morrison AFRC Institute of Animal Health, Compton, England	Consultant Bovine immunology	17/2/92–23/2/92
Dr. E. Anderson Veterinary Research Laboratory Harare, Zimbabwe	PCR technology for *Theileria* diagnosis	18/2/92–22/2/92
Dr. P. Ooijen IAEA Vienna, Austria	Collaborative research in diagnosis of trypanosomiasis	13/4/92–16/4/92
Dr. V.B. Carruthers Molecular Parasitology Laboratory Rockefeller University USA	Transfection technology and *in vitro* systems for trypanosome genetics	23/4/92–26/5/92
Dr. M. Parsons Seattle Biomedical Research Institute Washington, USA	Developmentally-regulated protein kinases of trypanosomes	4/5/92–10/5/92
Dr. D. Martinez IEMVT, Guadeloupe French West Indies	Lymph duct cannulation for studies of cowdriosis	7/5/92–18/6/92
Prof. J. Miller Louisiana State University USA	Analysis of DNA of small ruminants	20/5/92–3/6/92
Dr. M. Eisler Department of Statistics and Modelling Sciences Strathclyde University Scotland, UK	ELISA technology for trypanosomiasis	21/5/92–12/7/92

Name	Project	Dates
Mrs. E. Gault Department of Statistics and Modelling Sciences Strathclyde University Scotland, UK	ELISA technology for trypanosomiasis	21/5/92–12/7/92
Dr. M. Schutte University Hospital Utrecht, The Netherlands	Bovine immunoglobulin genes	10/5/92–29/8/92
Dr. R. Spooner, ABRL, Edinburgh Scotland	Collaborative bovine immunogenetics research	1/6/92–6/6/92
Dr. R.J. Pierce CIBP, Institute-Pasteur, Lille, France	Vaccine development strategy	11/6/92–12/6/92
Dr. H. Clevers, University Hospital, Utrecht, Holland	Genetic analysis of *Theileria parva*	9/7/92–15/7/92
Dr. Y. Steiger, Subhumid Zonal Site, ILCA, Mombasa, Kenya	Parasite identification	1/8/92–15/8/92 16/11/92–22/11/92
Dr. B. Allsopp Ondersterpoort Veterinary Institute, Transvaal, South Africa	Collaborative investigations of DNA probes for theileriosis	7/8/92–14/8/92
Dr. T. Van De Putte University of Louvain Belgium	Neonatal immunity of cattle	11/8/92–18/12/92
Dr. P. Maina University of Nairobi Kenya	Locum Veterinarian	2/12/91– 6/1/92 17/8/92–16/9/92
Prof. V.O. Anosa Ibadan University Nigeria	Pathology of trypanosome infection	30/8/92–30/11/92
Dr. B. Wilkie Ontario Veterinary College Guelph, Canada	Antibody avidity in trypanosome infections	1/9/92–28/2/93

Name	Project	Dates
Dr. D. Robertson, Department of Statistics and Modelling Sciences Strathclyde University Scotland, UK	Consultant Statistician	15/9/92–28/9/92
Dr. B. Goddeeris University of Louvain Belgium	Consultations on immunity to theileriosis	18/9/92–26/9/92
Dr. J.W. Hansen FAO Rome, Italy	FAO Visiting Scientist Editorial work on publication *Parasite Control*	1/10/92–9/10/92 27/11/92–1/12/92
Dr. E. Ronchi UNDP New York, USA	UNDP Consultant Scientific exchange	10/11/92–21/11/92
Dr. R. Brun Swiss Tropical Institute Basel, Switzerland	Assays for chemotherapy of trypanosomiasis *in vitro*	12/11/92–18/11/92
Ms S. Neals Through the Crawford Fund, Victoria Australia	CGIAR Editor	26/11/92–27/11/92