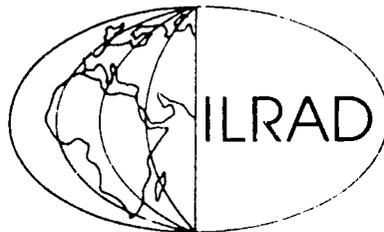


ILRAD 1994

Annual Scientific Report



International Laboratory  
for Research on Animal Diseases

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## PREFACE

We are pleased to present the collected scientific abstracts describing the research conducted by the International Laboratory for Research on Animal Diseases in 1994. As reference to the Table of Contents clearly indicates, ILRAD has been carrying out programmes of research on tick-borne diseases and trypanosomiasis, two disease complexes which severely limit livestock health and productivity in developing countries, particularly in sub-Saharan Africa. In recent years, these two long-standing research efforts have been effectively integrated with two newer programmes of research: ruminant genetics; and socioeconomic and environmental impact of these diseases and their control.

Although the individual reports are written as discrete abstracts, it will be evident on close reading that they represent elements of continuing research themes. Thus interested readers are often referred to earlier reports for some details that an abstract inevitably cannot contain. Similarly, it will become apparent that whilst ILRAD continues to seek to improve present control methods for these diseases, and to develop novel and sustainable means for the future, research is carried forward on a number of complementary fronts with the production of many intermediate research successes and of useful technologies.

The Tick-Borne Diseases Programme has three major elements. The first is to improve knowledge of the epidemiology of these diseases and of the organisms which cause them. This helps not only define the extent of the disease problem being addressed but in identifying the epidemiological tools required (such as appropriate diagnostic assays for the causative parasites). Such investigations also lay the groundwork for the application of future, new control methods, including vaccines, as they are developed. The second area is a focused investigation of parasite antigens which may provide the basis either of diagnostic assays or of vaccine formulations against the tick-borne diseases of livestock. Such investigations, as can readily be appreciated, require the application of molecular genetic and contemporary immunological assay techniques such as are used in equivalent medical studies of man or experimental investigations of the laboratory mouse. One of ILRAD's past strengths has been to develop knowledge of the ruminant immune system, and reagents to mark and characterize its component parts. The third element of the Tick-Borne Disease Programme is utilizing this capacity to develop vaccine procedures appropriate to the production of protective immune responses against these diseases in cattle. Much of this work focuses on the protozoan parasite *Theileria parva*, the causative agent of East Coast fever in cattle. However, the Laboratory's extensive expertise in the areas just described have been coupled with research conducted by other, collaborating institutes to tackle, through similar lines of research, other important tick-borne diseases of livestock with a global distribution.

In the case of trypanosomiasis, two major approaches are being followed. Again, improved epidemiological understanding of the disease complex is critical, both for the more effective deployment of existing control measures, principally chemotherapy, and to allow the development of technologies appropriate for national and regional bodies in Africa and elsewhere to mount their own programmes of control more efficiently. The second area (the T2 set of abstracts) describes research to

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investigate the host-parasite interactions in trypanosomiasis. Molecular differentiation of trypanosomes and the physiological and immune mechanisms that trypanotolerant, but not trypano-susceptible, cattle elaborate against trypanosome challenge are being investigated. Closely integrated with this research is the Ruminant Genetics Programme, which seeks to first mark and then identify the genes responsible for the trypanotolerance trait exhibited by the N'Dama, a breed of cattle indigenous to West Africa.

As with much of ILRAD's research the genetics studies have strategic implication. The markers and genetic mapping are applicable to the identification of other adaptive and production traits in ruminants. Simultaneously, of course, it provides the complementary genetic approach to the Laboratory's functional investigation of heritable disease resistance which holds out promise as a sustainable solution to animal trypanosomiasis.

The socioeconomics research has provided methodologies, computerized systems (such as economic models and geographic information systems) and data to better evaluate the cost (in economic, environmental and social terms) of the diseases that ILRAD is focussing upon. More accurate economic assessments of diseases provide the background for determining the most appropriate control measures to apply against these diseases in the future and, to some extent, the way in which any particular control measure should be applied to maximize its effect. Whilst the nature of these reports is evidently different from the reporting of molecular science, we hope interested readers will be stimulated to apply to the institute for more detailed reports (or the relevant publication) to gain a fuller understanding of this important component of the Laboratory's animal health research.

ILRAD was officially inaugurated in 1973 and opened a large part of its existing laboratory complex in Nairobi in 1978. It was always envisaged by the Consultative Group on International Agricultural Research (CGIAR)—the donor group which developed, sponsors and frames the work of seventeen International Agricultural Research Centres—that ILRAD's focussed scientific work was a component of a broader approach to the improvement of livestock productivity. In October 1993, the CGIAR decided that the time was appropriate to create a new livestock research entity, the International Livestock Research Institute (ILRI). ILRI will have a global mandate to improve the role livestock plays in sustainable agricultural systems in tropical developing countries. The new institute will subsume the research programmes of ILRAD and its sister CGIAR institute, the International Livestock Centre for Africa (ILCA). ILRI will commence operations from 1 January 1995 and so this document represents the final ILRAD Annual Scientific Report *per se*. ILRAD's current research, recent publications, scientific presentations at international meetings and collaborating visiting scientists—which are all listed in this report—yield a fairly accurate view of the Laboratory's scientific ethic, impetus and world-wide contacts in 1994. The Laboratory looks forward to linking this expertise and network of collaborators to wider animal production-related research in the new institute.

We fully expect that ILRI will issue Scientific Review Reports in the future but that the greater scientific and regional scope of the new institute (encompassing aspects of research on livestock nutrition and physiology, production systems analysis and economics—as well as animal health and genetics) will provide subject matter of interest to a wide range of scientists.

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In publishing the final volume of ILRAD's *Annual Scientific Reports*, we take this opportunity to thank all of ILRAD's donors, scientific visitors, collaborators and alumni for their support for the institute and its programmes.

We invite all our readers to share in the Laboratory's recent achievements. ILRAD is proud of its scientific and practical contributions to animal health research and disease control chronicled in its scientific publications, this *Annual Scientific Report* series since 1987, and the *Annual Reports* which have been published continuously since 1975. The Institute looks forward to continuing to contribute effective animal-health related research to the wider goals of ILRI in 1995 and beyond.

*P.R. Gardiner  
Information and Planning Officer  
International Laboratory for Research on Animal Diseases  
Nairobi, December 1994*

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# TICK-BORNE DISEASES

**E1a.1 Analysis of recombinant *Theileria parva* parasites****Scientists:** S. Morzaria, R. Bishop, A. Young, T. Dolan**Research Associates:** P. Spooner, F. Mwakima, R. Skilton

Cloned, recombinant *Theileria parva* parasites derived from elements of the Muguga/Uganda and Muguga/Marikebuni stocks have been isolated from animals experimentally co-infected with these 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. Sporozoite populations hybridizing with both oligonucleotides were considered to contain recombinant parasites. Cell lines containing cloned recombinant parasites were isolated after infection of bovine PBM at a ratio of less than 1 sporozoite/100 lymphocytes and limiting dilution cloning of the cells. Analysis of the cloned cell lines with anti-schizont monoclonal antibodies (MAbs) and several DNA probes confirmed the recombinant nature of the parasites and were consistent with the recombinants having acquired chromosomes from both parental stocks. Sporozoite stabilates of the cloned recombinant parasites were prepared after infection of the autologous animals and tick isolation. Cattle immunized with the Muguga/Uganda recombinant were resistant to challenge with a *T. parva* Muguga bulk stabilate, but breakthrough infections occurred on challenge with a *T. parva* Uganda stabilate. The immunological properties of the recombinant were thus similar to those of the *T. parva* Muguga parent.

Size polymorphic telomeric *SfiI* fragments were separated on CHEF gels, together with a series of internal chromosomal polymorphic markers. Piropasm DNA from the recombinant parasites was examined. This analysis indicated that several chromosomes in the recombinant parasites contained telomeric *SfiI* fragments apparently derived from one parent and internal chromosomal loci originating from the other parent.

**E1a.2 Identification and isolation of molecules involved in the interaction between *Theileria parva* and its mammalian host or tick vector****Research Fellow:** R. Janoo**Scientists:** R. Bishop, S. Morzaria, D. McKeever, A. Musoke, V. Nene, J. Stayaert, A. Young**Research Associates:** P. Spooner, E. Gobright

A project has been initiated to clone specific categories of molecules which are hypothesized to be involved in the interaction between *Theileria parva* and bovine lymphocytes. Two classes of molecules are currently under investigation. Firstly, pore-forming membranolytic proteins. These are likely to be involved as *T. parva* exits from a membrane bound compartment of host origin to subsequently lie free in the lymphocyte cytoplasm, secondly, proteins which contain tubulin binding or nucleating motifs. The latter class of proteins is of interest since it has been shown by electron microscopy that after exit from the membrane bound compartment *T.*

*parva* is surrounded by a network of microtubules. In both instances the initial approach to identification involves the use of a cocktail of redundant PCR primers designed to amplify evolutionarily conserved domains present in these classes of molecules.

### **E1a.3 Analysis of the *Theileria parva* genome**

**Scientists:** S. Morzaria, R. Bishop, V. Nene

**Research Associate:** E. Gobright

The main objective of the genome analysis of *Theileria parva* is to provide basic information on the molecular structure of the parasite. The research will in the long term enable the identification of genes related to important biological traits such as virulence, transmissibility, drug resistance and antigen diversity. Currently a low resolution map of the *T. parva* genome is available and a number of markers have been generated across the genome. As an initial step towards generating a high resolution map of the genome, attempts have been made to construct large DNA libraries. A PI library of the *T. parva* genome has been constructed but was found to be difficult to work with as DNA from the recombinants could not be amplified for further characterization. In order to circumvent this problem the recently developed bacterial cloning system for mapping and analysis of complex genomes has been exploited. The bacterial artificial chromosome (BAC) is based on *Escherichia coli* and its single copy plasmid F factor. The BAC system is capable of maintaining genomic DNA fragments greater than 300 kb with a high degree of structural stability. A *T. parva* genomic library of 600 recombinants of average insert size of 50 kb has been produced. In parallel, a cDNA library from *T. parva*, derived from an enriched population of schizonts, has been prepared in  $\lambda$ gt11. The library contains about 50% parasite sequences. Work has begun to array this library in microtitre plates and order the clones. Both the large DNA recombinants and the schizont cDNAs will ultimately be localized on the *Sfi*I fragments. The schizont cDNAs will also be sequenced and a database of the sequences established.

### **E1a.4 Characterization of the mitochondrial genome of *Theileria parva***

**Supervisors:** V. Nene, A. Musoke, S. Morzaria, R. Newbold\*

**Student:** G. Shukla

\*Brunel University, Middlesex, UK

The mitochondrial genome in *Theileria parva* is represented by a linear molecule of 7.1 kb. Most of the molecule in the subtelomeric region has been sequenced. By exploiting the cycle sequencing technique and the copy number of the mitochondrial DNA in total piroplasm DNA, another 250 bp of DNA sequence has been added to each telomeric end of the mitochondrial DNA. Synthetic oligonucleotides have been

used in Northern blots to confirm that at least the 3' half of LSU rRNA is fragmented. cDNAs coding for cytochrome oxidase I (*coxI*) and *coxIII* have been isolated and preliminary data suggest that the *coxI* transcript has alternative polyA processing sites. Techniques are being developed to determine the terminal sequences of transcripts originating from this genome. Attempts are being made to generate drug resistant mutants since one site of action of hydroxynaphthaquinones is at the level of cytochrome b. A comparison of the sequence of apocytochrome b from four different stocks of the parasite has therefore been made. This analysis has revealed a few point mutations about 15 residues downstream of a region predicted to be involved in ubiquinol oxidation. The drug sensitivity of these parasites *in vitro* will be examined. cDNA coding for dihydro-orotate dehydrogenase has also been isolated since this may be an additional drug target site.

**E1b.1 Antibody detection ELISA for *Theileria parva*****Scientists:** S. Morzaria, A. Musoke, V. Nene, P. Toye, T. Dolan**Research Associates:** J. Katende, C. Nkonge

Comparative analysis of four putative diagnostic antigens for *Theileria parva* has shown that the polymorphic immunodominant molecule (PIM) provides the highest sensitivity when used in the improved antibody detection ELISA. This antigen has been produced by *Escherichia coli* as a fusion protein with glutathione S-transferase (GST) molecule. The recombinant protein was purified using affinity chromatography on glutathione/sepharose and used to coat ELISA plates directly. The antigen has been tested in this format against a range of sera obtained from cattle experimentally infected with different cattle-derived stocks (Muguga, Marikebuni, Uganda, Boleni) and one buffalo-derived stock (7014). Additionally, the antigen has been evaluated using field sera obtained from areas of Kenya and Uganda for endemic East Coast fever but not from southern Africa where January disease occurs and non-endemic areas of West Africa, Great Britain, Sudan and Australia. The results of these studies have shown that the PIM-based antibody detection ELISA for *T. parva* has greater than 95% specificity. The test is awaiting field validation before its adoption for wider use.

**E1b.2 Antibody detection ELISA for *Theileria mutans*****Scientists:** S. Morzaria, A. Musoke, V. Nene, T. Dolan**Research Associates:** J. Katende, R. Skilton

The recombinant 32 kDa antigen (p32), selected as a candidate antigen for use in a *Theileria mutans* antibody detection ELISA, has been characterized and a number of alleles of the gene encoding the antigen have been identified. Two of these alleles, clone 21 and clone 23, have been expressed as fusion proteins with glutathione S-transferase (GST) and compared for sensitivity and specificity in an antibody detection ELISA. The GST/clone 23 has been found to show higher sensitivity and specificity than the GST/clone 21. The recombinant protein expressed by the allele GST/clone 23, representing full length p32, has been evaluated using sera from tick-transmitted *T. mutans* infections in cattle and areas of Uganda and Kenya either endemic or non-endemic for *T. mutans*. The test shows over 99% sensitivity and 95% specificity. The antigen is awaiting testing with sera collected from areas in West and Southern Africa where *T. mutans* is endemic. Studies on epitope mapping using experimental sera have shown that GST/clones 21 and 23 express epitope polymorphisms (see abstracts E2c.1 and 2). Therefore studies are in progress to map epitopes using field sera. The results may open up the possibility of using defined epitopes rather than the whole protein for antibody detection in the ELISA.

**E1b.3 Antibody detection ELISA for *Anaplasma marginale*****Scientists:** S. Morzaria, A. Musoke, V. Nene, T. Dolan**Research Associate:** J. Katende

The gene for the 19/23 kDa protein, identified as a candidate antigen for use in an *Anaplasma marginale* antibody detection ELISA, has been obtained through collaboration with the Washington State University and expressed in *Escherichia coli* as a fusion protein with GST using the pGEX vector. The recombinant protein has been used in the antibody detection ELISA system, similar to that used for *Theileria parva* and *T. mutans*, and evaluated for specificity. The recombinant antigen has been found to detect *A. marginale* specific antibodies from experimentally infected cattle and does not cross-react with sera raised against *Cowdria ruminantium*. Tick-transmission experiments are in progress to collect sequential sera from experimentally infected cattle for evaluating the specificity of the test.

**E1b.4 Antibody detection ELISA for *Babesia bigemina*****Research Fellow:** N. Tebele**Scientists:** S. Morzaria, A. Musoke, V. Nene, T. Dolan**Research Associate:** J. Katende

A 200 kDa antigen of *Babesia bigemina* has been identified as a candidate antigen for use in the antibody detection ELISA system. 3.8 kb of the coding sequence of the gene of this antigen has been expressed in *Escherichia coli* as a fusion protein with GST and is being evaluated using a range of sera. The antigen reacted with sera from cattle following infection with a sporozoite stabilate of *B. bigemina*. In addition, 21% of 200 sera collected from cattle in Tasmania, where *Babesia* spp. are thought not to occur, showed cross-reactivity with the recombinant antigen. In an attempt to remove the cross-reacting epitopes, epitope libraries from the DNase-digested fragments of the gene expressing the 200 kDa protein were constructed in  $\lambda$ gt11. The libraries containing the DNA fragments between 100–500 bp were immunoscreened with hyperimmune sera from cattle and one strongly reacting clone of 190 bp was isolated, recloned in the pGEX vector and expressed as a fusion protein. The fusion protein has been evaluated with a limited number of sera in Western blots and antibody detection ELISA. The smaller recombinant protein has the same specificity as the protein expressed by the 3.8 kb coding sequence and it cross-reacts with only 7% of the 200 sera from Tasmania. Studies are in progress to test the smaller fusion protein derived from the 200 kDa antigen against a number of other reference sera to determine its specificity.

**E1b.5 DNA probes for *Theileria*****Scientists:** S. Morzaria, R. Bishop**Research Associate:** R. Skilton

A number of DNA probes are available to discriminate among different *Theileria* species. These are based on parasite specific repetitive sequences and small and large subunit ribosomal RNA genes. These DNA sequences have been successfully used as radioisotopically labelled probes to identify various species of *Theileria*. Although these probes are highly specific, the need to use radioactivity for labelling and to prepare DNA from a large number of parasites limit their use. Therefore, attempts are being made to design primers for use in a PCR-based system with samples collected directly from the infected host. One approach being considered is multiplex PCR amplification using a cocktail of species-specific primers and visualization of ethidium bromide stained amplicons under UV. Initial studies have focused on defining and optimizing conditions necessary for PCR amplification of *T. parva* target sequences using whole infected bovine blood. Employing primers which define a 405 bp fragment of the *T. parva* repetitive sequence (*TpR1*), it has been determined that amplicon production can be achieved, without pretreatment of whole blood, using Tricine or Tris-based buffers. Potassium chloride in the PCR buffer is essential for amplicon production from blood collected in heparin but not from blood collected in EDTA. Freeze/thawing of the blood sample appears to enhance amplicon production. *Taq*, *Tth* or *Tfi* DNA polymerases can be used to amplify DNA from whole blood. Up to 20% v/v whole blood is tolerated in the PCR reaction volume of 25  $\mu$ l. Using these conditions and *TpR1* primers, as few as four copies of the target sequences of *T. parva* from the whole blood have been detected. This represents approximately 1/25th of the DNA derived from one parasite genome, thus providing a test with very high sensitivity. Studies are in progress to develop a set of primers that can be used to differentiate various *Theileria* species by PCR-based methodologies targeting rRNA sequences.

**E1c.1 Analyses of factors influencing *Theileria parva* infections in *Rhipicephalus appendiculatus* ticks****Scientists:** A. Young, T. Dolan, S. Morzaria, A. Sherriff\*, G. Gettinby\***Research Associates:** F. Mwakima, J. Scott

\*University of Strathclyde, UK

Further analysis of a large tick transmission database has been undertaken using transformed infection variables (arcsine square root for prevalence and the logarithm for intensity and abundance) by the General Linear Interactive Model (GLIM) using Type I sums of squares analysis. It was found that transformation increased the sensitivity of the analysis. Six statistical models were constructed to explain the variation in infection variables (prevalence, abundance and intensity) in male and female ticks which accounted for between 31 to 39% of the variation in the infection variables. While these cannot be considered to be very accurate predictive models, they represent an improvement over previous knowledge. As shown elsewhere, a high proportion of variability is likely to be due to variation in the susceptibility in tick vector populations which was not explored in these analyses. Type III sums of squares analysis was also performed on the data and it was found that piroplasm level and the month of year were important sources of variation as in the Type I analysis. In general Type III analysis was not as useful as Type I analysis.

Using the information in the original database and the results of the foregoing analyses, a new standard procedure for infecting ticks was developed during the last year. Cattle were infected with 1/20 dilution of a *Theileria parva* stabilate and the infection was prolonged by treatment with oxytetracycline. In a group of 64 cattle it was found that tick batches could become infected from day 13 after infection and infection levels showed a linear increase in infection up to day 17 when more variable levels occurred. Infection in cattle decreased from day 20. Using this information, tick application to cattle was redesigned so that large numbers of engorged ticks were obtained from infected cattle on days 17 to 19. This resulted in a much more efficient production of highly infected tick batches which has facilitated the supply of sporozoites to the institute's scientists. In addition, it was found that ticks that engorged on day 14 and 15 after infection gave predictably lower infections which are required for the challenge of immunized cattle. The longitudinal statistical analysis of this database has confirmed these observations.

**E1c.2 Further analyses of the transmission dynamics of *Theileria parva* by *Rhipicephalus appendiculatus*****Scientists:** A. Young, G. Medley\*, B. Perry, S. Morzaria, T. Dolan**Students:** H. Ochanda, C. O'Callaghan\*, A. Ward\*\*Department of Biological Sciences  
University of Warwick, UK

Studies on the transmission of *Theileria parva* by larvae and nymphae and adult male and female *Rhipicephalus appendiculatus* were completed during the year. It was found that nymphae fed as larvae on *T. parva*-infected cattle had a lower prevalence and a much lower intensity and abundance of infection than either males or females fed as nymphae. This difference was more pronounced if carrier cattle were used as a source of infection rather than acute infections. Larval to nymphal transmission was found to be inefficient if a stock of *T. parva* was used, such as Boleni, which did not produce high piroplasm parasitaemia.

It was also found in laboratory studies that *R. appendiculatus* stocks had different susceptibilities to *T. parva* infection which was not necessarily the same for different stocks of *T. parva*. This could substantially affect the epidemiology of *T. parva* infection in different geographical zones, particularly if parasite stocks are being used widely within the region for immunization. Further studies are required to confirm these potentially important observations.

Extensive studies on the survival of *T. parva* in the salivary glands of *R. appendiculatus* were completed during the year. In highly infected ticks, the salivary gland infection in adult ticks collapsed rapidly under various temperature regimens in the laboratory, some of which were representative of field conditions prevailing within the distribution of *T. parva*. However, the ticks in many cases survived as long as they were exposed to field conditions. Under field conditions at ILRAD, the infections survived much longer, nearly as long as the ticks themselves, and reduction in salivary infection occurred much later than in ticks kept under laboratory conditions. Survival of salivary gland infection was found to be density dependent as infection died off more rapidly in highly infected ticks than in those with low infections. These results have been analysed using the General Linear Model analysis and statistical models constructed.

The preliminary *T. parva* transmission model (Medley, Perry and Young, 1993, *Parasitology* 106, 251–264) which used data from an endemically stable situation in the Trans-Mara District, Kenya, has been developed further during the year using the new data reported above so that it can be applied in a wider range of conditions.

**E1c.3 Selection of lines of *Rhipicephalus appendiculatus* with greater or lesser susceptibility to *Theileria parva* infection****Scientists:** A. Young, R. Dolan\*, T. Dolan**Research Associate:** F. Mwakima**Technicians:** S. Mwaura, G. Njihia, M. Muthoni

\*Stockwatch, Nairobi, Kenya

The production of crosses between full sib groups of *Rhipicephalus appendiculatus* which showed low and high infection levels of *Theileria parva* has been completed so that selection of lines with greater or lesser susceptibility to *T. parva* infection can be made. From the original experiments reported last year, three groups of full sibs were selected from the *R. appendiculatus* Muguga and Kiambu stocks. The selection groups were three full sibs with low *T. parva* infections from the Muguga stock, three full sibs with high infections from the Kiambu stock and three full sibs with low infections from the Kiambu group.

For each of the selection groups, three matings were obtained from the nine combinations and the resultant engorged females were allowed to lay eggs. From each selection group, 27 offspring batches were obtained which were fed on *T. parva*-infected cattle as nymphae together with a sample from the unselected population as a control. The *T. parva* infection in 27 groups of ticks for each of three selection groups is now being assessed in the resultant adult ticks. After this assessment it is hoped that lines of *R. appendiculatus* with lower and higher susceptibility to *T. parva* infection can be established.

**E1c.4 Effect of diapause in adult *Rhipicephalus appendiculatus* populations on the transmission of *Theileria parva*****Research fellow:** H. Ochanda**Scientists:** A. Young, S. Morzaria, T. Dolan, W. Mazhowu\*, R. Pegram\*, D. Kariuki†**Research Associate:** F. Mwakima**Technicians:** S. Mwaura, G. Njihia

\*Veterinary Research Laboratory, Harare, Zimbabwe

†National Veterinary Research Centre (KARI), Muguga, Kenya

Over the last three years extensive experiments have been carried out to investigate factors controlling diapause in *Rhipicephalus appendiculatus* and *R. zambeziensis* populations in Africa. Diapause has a major effect on the transmission of *T. parva* in areas where it occurs in that seasonal transmission of *T. parva* is seen. In laboratory experiments, strong diapause has been observed in *R. appendiculatus* colonies established from ticks collected from Zambia and Zimbabwe as judged by their feeding behaviour. Ticks exposed to total darkness and short day length (10 hr light, 14 hr dark) showed strong diapausing behaviour while ticks exposed to long day

length (14 hr light, 10 hr dark) did not. When ticks had entered diapause, it was found that exposure to a longer photoperiod for about three weeks was required before diapause behaviour was broken. The critical photoperiod for the termination of diapause appeared to be over 13 hr. Ticks from Kenya and Uganda kept under the same conditions as ticks from southern Africa did not enter diapause as judged by feeding behaviour. In the case of *R. zambeziensis*, inconclusive results were obtained which indicate that the tick species did not enter diapause.

Experiments on the manifestations of diapause under field conditions in Zimbabwe and Kenya have also been carried out over the last three years. *Rhipicephalus appendiculatus* ticks were obtained by collection of engorged females from cattle in the field in Kiambu District of Kenya, Eastern Province of Zambia and Western Mashonaland in Zimbabwe in January/February 1992. The resultant larvae were exposed to natural conditions at ILRAD, Kenya, and the Veterinary Research Laboratory, Harare, Zimbabwe. The hardened larvae were applied to rabbits and allowed to moult after which the nymphae were exposed to natural conditions on both sites. The hardened nymphae were fed on rabbits and these were exposed immediately as engorged nymphae in nylon columns under field conditions at ILRAD and Harare. After the nymphae had moulted into adults their behaviour in the nylon columns was observed.

In Kenya, it was found that Kenyan ticks were active throughout the observation period (eight months) and both Zambian and Zimbabwean ticks showed a much lower activity. Columns were sampled every month and ticks were applied to rabbits to observe their feeding behaviour. Kenyan ticks showed a rapid and high attachment rate to rabbits and a high female engorgement rate, in contrast to the Zambian and Zimbabwean ticks which showed a slow and low attachment rate. This experiment was repeated in the following year using the same tick stocks but with the next generation. Similar results were obtained.

Similar experiments were carried out at Harare. Over the two exposures there was no evidence that the Kenyan ticks had entered diapause, either by their activity or feeding success on rabbits, but both the Zambian and Zimbabwean ticks showed evidence of strong diapause as judged by their behaviour in the columns and their feeding behaviour on rabbits. However, in both years the diapause was terminated in December as judged by these criteria. This indicated that the critical photoperiod for termination of diapause was over 13 hours.

The conclusion from these studies is that there are probably three populations of *R. appendiculatus* in Africa: (1) an equatorial diapausing adult population which occurs in Kenya, Northern Tanzania, Uganda, Zaire and Southern Sudan, (2) a southern African population which shows strong diapause in adult ticks which occurs in Southern and Central Province of Zambia, Zimbabwe and South Africa and (3) an intermediate population which has the ability to enter diapause or not depending on the time of nymphal moult which may occur in Eastern and Northern Provinces of Zambia, Malawi and southern Tanzania. While modelling the population dynamics of *R. appendiculatus* in the equatorial and southern Africa population of ticks may now be straightforward, more research is required on the diapause in the intermediate population. The effects of diapause on the transmission of *T. parva* is now being investigated.

**E1c.5 Optimization of the feeding of *Rhipicephalus appendiculatus* nymphae *in vitro* for the transmission of *Theileria parva*****Scientists:** S. Waladde\*, A. Young**Research Associate:** F. Mwakima**Technicians:** S. Mwaura, G. Njihia

\*International Centre of Insect Physiology and Ecology, Nairobi, Kenya

An apparatus for artificial feeding of *Rhipicephalus appendiculatus* nymphae was modified to improve their feeding performance. Heparinized blood was contained above a treated artificial membrane while the ticks attached to the lower surface of the membrane. The blood was replaced twice daily. The feeding apparatus was incubated at 37 °C in an atmosphere of 3% CO<sub>2</sub> concentration and a relative humidity stabilized at 75–80%. The stabilization of humidity proved to be particularly important for the improved feeding of ticks *in vitro*. Under these conditions, 91% of the engorged nymphae attained a mean weight of between 6 and 11 mg and the mean percentage of adult ticks which moulted from the artificially engorged nymphae was 94%. When this system was used to feed nymphal ticks on blood infected with *Theileria parva* piroplasms, the mean prevalence of infection in female and male ticks was 86 and 54% respectively. The feeding performance and *T. parva* infection levels were comparable to those of nymphal ticks fed on the blood of donor cattle infected with *T. parva*. The apparatus has the potential for adjustment to suit artificial feeding needs of other species of ixodid ticks for investigations of tick/pathogen relationships. It has been possible to use cryopreserved rather than fresh blood in this method. The use of cryopreserved blood may allow the isolation, by late tick pick up, of *T. parva* from the field and from laboratory animals. In addition this would improve the practicability of the *in vitro* feeding method. The technique is now being used to determine the direct relationship of piroplasm parasitaemia on the infection levels in ticks.

**E1c.6 Probability estimates to assess infection in *Theileria parva*-infected ticks****Scientists:** A. Young, G. Gettinby\*, I. McKendrick\*, S. Morzaria, T. Dolan**Research Associates:** J. Scott, F. Mwakima

\*University of Strathclyde, UK

Batches of *Rhipicephalus appendiculatus* ticks were obtained with reproducibly low infection levels. This was achieved by applying nymphal ticks to cattle which had been infected with *Theileria parva* Muguga stabilate diluted at 1/20 so that the ticks become engorged on days 14 and 15. These ticks were required to provide a low but even challenge, simulating field challenge, for evaluating the status of cattle immunized against this parasite. The requirement was for all cattle to become infected but also to receive the minimum challenge judged by the number of infected acini in the

ticks. A Monte Carlo method computer programme has been developed to predict the challenge of cattle by a certain number of ticks from a particular tick batch. The requirement was to determine the proportion of cattle in a group that would become infected and to predict the variation in the number of infected acini received by the cattle in the group. A consistent relationship was found between tick numbers, the maximum and minimum number of infected acini and the number of cattle which became infected. The Monte Carlo programme has been rewritten to provide further information on the nature of the predicted challenge by sub samples from batches of infected ticks.

### **E1d.1 Characterization of important immunizing stocks of *Theileria parva***

**Scientists:** T. Dolan, R. Bishop, S. Morzaria

**Research Associate:** P. Spooner

**Technician:** J. Kiarie

This work has been carried out in conjunction with the FAO Regional Vaccine Production Facility (Malawi), the national projects in Zambia and Zimbabwe and projects funded by Belgium and the FAO.

Monoclonal antibodies and DNA probes have been used to characterize isolates and stocks of *Theileria parva* and to provide a unique fingerprint for any particular parasite. However, these characterization methods have not been shown to correlate with the immunizing capacity of the parasite which has to be determined in cross-immunity studies. In order to provide a more formal definition of stocks for use in field immunization, the development of monoclonal antibody (Mab) and DNA profiles of reference and working stabilates is required. Based upon a range of characterization reagents used both for laboratory stock identification and for field studies, a set of reagents has been selected and applied to define the components of the Malawi *T. parva* trivalent vaccine, (Muguga, Kiambu 5 and Serengeti-transformed) and the Katete (Eastern Zambia), Boleni (Zimbabwe) and Marikebuni (Kenya) stocks. These are the major immunizing stocks being used in the region.

Monoclonal antibody profiles are determined using MAbs 1, 2, 3, 4, 7, 10, 12, 15, 20, 21, 22 and 23. The *T. parva* DNA probes that provide the most useful descriptions of the parasites are the *Tpr* repetitive sequence, a telomeric probe, a multicopy sequence with protein coding potential, LA6, and an M13-homologous sequence. The relevance of these characterization reagents will be considered by a committee set up to determine standards for live vaccines for tick-borne diseases. If these reagents are adopted, it is intended to transfer the reagents to the appropriate laboratories. The reagents will have additional value in the characterization of breakthrough parasites following field immunization and for new immunizing stocks. Further work in this area will be directed to the development of reagents that are unique markers for immunizing stocks, probably based upon regions of the *Tpr* genes, in order to follow the spread of the immunizing parasite following its introduction into a cattle population in a new area.

This work will contribute to the determination of laboratory characterization methods by a committee set up in 1991 by FAO/OAU/ILRAD.

**E2a.1 Sequence analysis of the p67 gene from different stocks of *Theileria parva*****Scientists:** V. Nene, S. Morzaria, A. Young**Research Associate:** E. Gobright**Technicians:** S. Wanyoni, A. Kaushal

All cattle-derived stocks of *Theileria parva* that have been analysed encode the vaccine candidate antigen of *T. parva* sporozoites, p67, identical in sequence to that found in the Muguga stock. The p67 sequences of buffalo-derived parasites 7014, 7013, 7344 and Zimbabwe 96 have been established and are different from each other and from the p67 of the Muguga stock. All the buffalo parasites contain the 129 bp DNA insertion first described in stock 7014. From this limited study it is possible to categorize the p67 types as follows: Type 1: Muguga p67—all cattle-derived parasites; Type 2: like Type 1 with point mutations and peptide insert at position 303/304 (buffalo 7014 and 7344); Type 3: like Type 2 with polymorphisms between positions 230-350 and 440-520 (buffalo 7013); and Type 4: like Type 3 with polymorphisms between 170-300 and 440-520 and an additional peptide insert at about position 170 (Zimbabwe 96). The p67 gene of buffalo 7014 is the most similar to the p67 antigen gene of the Muguga stock and the p67 gene of Zimbabwe 96 the most divergent.

Only partial p67 sequences of buffalo-derived parasite DNA from South Africa, Kruger National Park 2 and Hluhluwe, have been established because the extreme primers used to amplify p67 from the other parasites do not work on these DNAs. The available data suggest that the p67 sequence will fall into two new types. Thus it would appear that buffalo-derived *T. parva* contain polymorphic p67 sequences and it is likely that an extensive survey would have to be carried out to define the number of different buffalo-derived p67 types that exist.

**E2a.2 Characterization of molecules involved in *Theileria parva* sporozoite entry into lymphocytes****Scientists:** J. Syfrig, A. Musoke, J. Naessens, V. Nene, J. Steyaert**Technician:** J. Gachanja

An assay for analysis of the binding of *Theileria parva* sporozoites to peripheral blood lymphocytes (PBL) has been established. Bound sporozoites are detected by a monoclonal antibody to the sporozoite antigen p67, followed by a fluorochrome-labelled antibody against mouse immunoglobulin and subsequent analysis in a flow cytometer.

To determine whether p67 binds to PBL, this assay was used to analyse the inhibitory capacity of p67<sub>622</sub>, a recombinant, soluble part of p67. No decrease in sporozoite binding was detected by preincubating PBL with increasing concentrations of p67<sub>622</sub>. Correspondingly, no direct binding of p67<sub>622</sub> to PBL was detected using a variety of methods including direct and indirect labelling of p67<sub>622</sub> as well as coupling it to magnetic beads. However, since p67<sub>622</sub> is not recognized by an inhibitory

monoclonal antibody to a conformation-dependent epitope of the native p67, this result does not exclude the involvement of p67 in sporozoite binding to PBL.

Flow cytometry was also used to test the inhibitory capacity of a panel of 40 monoclonal antibodies against PBL surface molecules. Significant inhibition was only produced by antibodies against major histocompatibility complex (MHC) class I molecules confirming previous results. Experiments using different concentrations of antibody showed a clear difference between two antibodies to MHC class I; IL-A 19 with which inhibition is closely correlated to its binding to PBL, and IL-A 88 which only inhibits in significantly higher concentrations than necessary for binding. Since the affinities of both antibodies for their epitopes are in the same range, this result suggests that the IL-A 19 epitope is at the sporozoite binding site of the MHC class I molecule or very close to it, whereas the IL-A 88 epitope is located more distantly.

Co-precipitation experiments utilizing the sporozoite ligand(s) for MHC class I might reveal an important component of a potential vaccine against *T. parva*.

### **E2a.3 High level expression of soluble p67 as a secretory protein in *Escherichia coli* and purification of the gene product**

**Scientists:** J. Steyaert, V. Nene, A. Musoke

**Research Associate:** E. Gobright

**Technicians:** J. Nyanjui, S. Wanyonyi, L. Gichuru

High level expression of the surface exposed fragment of p67 of *Theileria parva* as a secretory protein in *Escherichia coli* has been established. A gene that codes for p67, fused at its N-terminal end to the *E. coli* *phoA* secretion signal, was constructed and the chimaeric gene was put under the transcriptional control of the IPTG inducible *P<sub>tac</sub>* promoter. A translational stop codon was engineered into the gene after residue 622, deleting the C-terminal membrane anchor region of the gene product. This construct expressed about 2 mg p67<sub>622</sub>/litre of cell culture.

To improve the expression levels of this initial construct the polylinker sequence between the secretion signal and the antigen encoding region was removed. Using site directed mutagenesis with mixed oligonucleotides, a set of clones encoding Ala<sup>^</sup>Ala XXX Ala Gly Asp (where <sup>^</sup> marks the signal sequence processing site) were generated and screened for antigen expression on immunoblots using MAb ARIII 22.7. One clone, encoding Ala<sup>^</sup>Ala Arg Ala Gly, was selected. Cells transformed with this construct grew to high densities and produced an estimated 40 mg/litre of soluble p67<sub>622</sub>.

A purification scheme was developed for unfused p67<sub>622</sub> involving an ammonium sulphate precipitation step (60%), an anion exchange step at pH 6.0 (Mono Q 10/10, Pharmacia), a reversed phase step (Phenyl Superose 10/10, Pharmacia) and a gel filtration step (Superdex 200 16/60, Pharmacia). Following this scheme, about 50 mg of antigen could be purified from 2 litres of bacterial culture within a week. The final material is soluble under physiological conditions and only minor degradation has been observed during the various purification steps. The immunogenicity of this preparation is being evaluated in cattle.

To allow easier purification of p67<sub>622</sub>, two different constructs were made: (1) a N-terminal fusion with the RNase A-derived S peptide and (2) a C-terminal fusion with a peptide showing high affinity for streptavidin. Although both constructs expressed reasonable quantities of the antigen, purification of the respective products on RNase S and streptavidin columns failed. Finally, a construct that encodes 635 residues of p67 followed by six histidines (p67<sub>635</sub>) was made. This fused antigen binds with reasonable affinity and high specificity to chelating sepharose FF (Pharmacia), charged with Zn<sup>++</sup>. The purification of p67<sub>635</sub> using immobilized metal affinity chromatography (IMAC) is being optimized. This should allow purification of large quantities of recombinant P67<sub>635</sub> in a single step procedure.

#### E2a.4 Biochemical characterization of *Theileria parva* sporozoite proteins

**Scientists:** Y. Yagi, V. Nene, A. Musoke

**Technician:** D. Lugo

*Theileria parva* sporozoite antigens which are recognized by bovine polyclonal antisera and murine monoclonal antibodies had been identified previously and the genes encoding these proteins isolated, characterized and expressed in *Escherichia coli*. In particular, three molecules, p67, p85 and p104, were of interest as components of recombinant vaccines or diagnostic tests. A fourth antigen, p32, was included in the study since it is an abundant antigen of piroplasms. Little information was available on the native parasite antigens and procedures were developed to analyse the biochemical properties of these molecules using specific MAbs as probes.

Antigen p32 was shown by immunoblotting to be expressed by sporozoites, schizonts and piroplasms, p85 was expressed by sporozoites and schizonts, while p67 and p104 were expressed by sporozoites only. Biotinylation of whole sporozoites followed by immunoprecipitation revealed that p32, p67 and p104 were accessible to the labelling reagent indicating that these antigens are close to or exposed at the surface of sporozoites. All four molecules were found in the detergent Triton X-114 phase, suggesting their hydrophobic nature and perhaps a membrane association. p32 and p67 were also present in the aqueous phase before detergent treatment.

The antigens were examined by 2-dimensional gel electrophoresis and p32 was shown to have a pI of 7.2, p67 a pI of 6.0, p85 a pI of 4.0 and p104 a pI of above 7.5. Efforts to determine whether these antigens or other sporozoite molecules contained carbohydrate sidechains or GPI anchors were unsuccessful. It is unclear whether this failure was due to a lack of these types of post-translational modifications or a lack of sensitivity of the techniques used. However, since these techniques detected the presence of sugar residues on recombinant p67 expressed by insect cells, it is likely that the native parasite molecules are not extensively modified. Experiments to determine whether p67 has protease activity were not possible to interpret because of a high level of contaminating enzyme activity from tick salivary glands.

Together, these results suggest that post-translational glycosylation of sporozoite molecules, if it exists, is minimal. The implication that p104 and p32 are at

or close to the surface of the sporozoite needs to be confirmed since these antigens may have vaccine potential. All four antigens are hydrophobic in nature but p32 and p67 also exist as soluble forms. The pI of p85 was of interest since the antigen did not exhibit the charge heterogeneity of p85 expressed by schizonts and was not labelled by biotin. These results may limit the potential of p85 as a vaccine antigen.

### **E2a.5 Isolation of the cDNA encoding the polymorphic immunodominant molecule (PIM) of *Theileria parva* Muguga by eukaryotic expression cloning in COS cells**

**Scientists:** P. Toye, M. Metzelaar\*, P. Wijngaard\*, J. Roose\* H. Clevers\*

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Previous attempts to clone the DNA encoding PIM by immunoscreening a *Theileria parva* piroplasm genomic DNA library expressed in  $\lambda$ gt11 were unsuccessful. Therefore a eukaryotic screening procedure was modified to allow cloning of cDNA encoding antigens irrespective of their subcellular site of expression. A cDNA library of *T. parva* (Muguga)-infected bovine lymphocytes was transiently transfected into COS cells. The transfected cells were fixed *in situ* with methanol and incubated with a mixture of anti-PIM MAbs 4 and 5. Reacting cells were located by subsequent additions of horse radish peroxidase-linked anti-mouse IgG and the chromogenic substrate 9-amino, 3-ethylcarbazol (0.02% in 0.1M sodium acetate pH 4.8 with 0.1% H<sub>2</sub>O<sub>2</sub>). Seven positive cells were identified in the population of transfected COS cells. Plasmid DNA was recovered from each positive cell by scraping the cell contents from the dish and extracting the DNA. After transformation of *Escherichia coli* MC1061/p3 by electroporation, plasmid DNA was prepared from individual *E. coli* colonies and retransfected into COS cells. Positive reactions with MAbs 4 and 5 were observed in COS cells transfected with plasmid DNA from one colony. This plasmid consistently induced the expression of proteins in COS cells which reacted with several anti-PIM MAb and polyclonal antisera and was therefore believed to contain cDNA encoding PIM.

Sequence analysis of the putative PIM-encoding plasmid revealed a cDNA insert of 1715 bp containing a single open reading frame of 480 amino acids, which includes an ATG translation start site at bp 244 and an in-frame stop codon at bp 1684. There is a tetrapeptide repeat, Gln-Pro-Glu-Pro tandemly reiterated five times beginning with amino acid 249. The sequence was found to be almost identical to that of the QP protein of *T. parva* (Baylis *et al.*, *Molecular Biochemical Parasitology*, 1993, 61: 171), except that the latter cDNA contains an additional 28 bp at the 5' end, a slightly longer poly (A) tail (19 residues as opposed to 12), and a cytosine rather than a guanine at position 880, and thus a glutamine at amino acid 213 as opposed to the glutamic acid, predicted in the sequence deduced at ILRAD. The predicted mass of the protein is 52386.59 Da.

**E2a.6 Isolation analysis of cDNA encoding the polymorphic immunodominant molecule (PIM) antigen from a buffalo-derived *Theileria parva*, and sequence comparison with cDNA of PIM of *T. parva* Muguga****Scientists:** P. Toye, V. Nene, P. Wijngaard\*, M. Metzelaar\*, H. Clevers\***Research Associate:** E. Gobright**Technicians:** J. Nyanjui, S. Wanyonyi\*Department of Clinical Immunology, University Hospital  
Utrecht, The Netherlands

The polymorphic immunodominant antigen (PIM) of a *Theileria parva* stock derived from buffalo 7014, as determined by immunoblotting, is larger than that of *T. parva* (Muguga), the Mr of the proteins being 112,000 and 90,000, respectively. The cDNA from a cell line infected with the buffalo-derived parasite was isolated and sequenced to determine the molecular basis of this size difference and the sequences of the two cDNAs were compared. The cDNA was isolated by colony hybridization using the PIM Muguga cDNA as a probe from a library made from the cell line, T19.4 TpL, derived by infecting a cloned T cell line with sporozoites of the buffalo-derived parasite stock, 7014. The resulting plasmid was transfected into COS cells which subsequently reacted with several anti-PIM MAbs and antisera.

Sequence analysis revealed that the 7014 cDNA insert was 1894 bp long with a single open reading frame of 543 amino acids. The predicted molecular mass of the protein was 60,023.42 which, as for the Muguga molecule, is less than that estimated from polyacrylamide gel electrophoresis.

When the sequences of the two PIM cDNAs were compared, considerable homology was found between the 5' and the 3' ends of the respective inserts. Thus, there was 97.5% identity at the nucleotide level between the initial 441 bp of the 7014 cDNA and bp 16-456 of the Muguga insert. This region includes 228 bp of 5' untranslated region. The terminal 662 bp of the 7014 sequence showed 96.8% identity at the nucleotide level to the corresponding region in the Muguga sequence. These identities were reflected at the amino acid level as 94.4% and 95.2% for the initial 71 amino acids and the terminal 208 residues, respectively. The central regions of the molecules showed identities of only 64% and 50% at the DNA and amino acid levels, respectively.

Within the central variant regions of the respective molecules, short repeated sequences were found. Although there is considerable sequence divergence between the Muguga and the 7014 molecules, there are some regions of identity scattered through these portions, most notably in the repeat regions. These repeats in some instances overlap, and are considered to be of three types. Firstly, there are two slightly different tetrapeptide repeats, which we have nominated as Type I repeats. The first has the sequence QPXP (X being E, Q, T, R or G), which occurs 11 times in Muguga and nine times in 7014, with no QPRP sequence in the latter. This group of repeats includes the QPEP motif which is tandemly repeated five times in the Muguga PIM. There is no comparable sequence in the 7014 PIM antigen, although it does contain the sequences QPQP QPQP QPEP and QPQP QPEP QPEP. The second tetrapeptide repeat is QPXQ, (X being D, I, Q, S, T, or V), which occurs ten times in Muguga and nine times in 7014.

Type II repeats contain three consecutive glutamine residues within a nine amino acid repeat, QPVDQQQPV, which is found four times in the 7014 sequence. A similar sequence, QPVYQQQPV, is present only once in the Muguga cDNA.

Type III repeats contain four consecutive glutamine residues within a variable repeat sequence, although one glutamine residue has been replaced by a histidine in one of the Muguga repeats. Thus, there is a 22 amino acid sequence, QPDQPEDQQGQQQPLDQPTGQ, present twice in the 7014 cDNA, and the 16 amino acid sequence, DQ(Q/H)QQPTQGDTSGQQG, imperfectly repeated in the Muguga cDNA.

### **E2a.7 Sequence analysis of the polymorphic immunodominant molecule (PIM) cDNA isolated from *Theileria parva* sporozoites and of a genomic clone**

**Scientists:** V. Nene, R. Bishop, P. Toye

**Research Associates:** E. Gobright, R. Skilton

**Technician:** S. Wanyonyi

To establish whether the sequence of the polymorphic immunodominant molecule (PIM) expressed by the sporozoites of *Theileria parva* was similar to that expressed by the schizont stage of the life cycle, cDNA encoding the PIM open reading frame was derived by PCR technology from RNA isolated from infected tick salivary glands. Sequence analysis indicated that the sequence was identical to that isolated from the schizont.

The gene encoding the PIM antigen was isolated by colony hybridization from a library of *T. parva* (Muguga) genomic DNA prepared from piroplasms and inserted into pBluescript. Sequence analysis of the PIM gene showed the presence of two introns, of 55 bp and 61 bp respectively, in the 3' conserved region. Both introns were preceded by the sequence AAAG and contained the sequences GTAAT and TTAG at their 5' and 3' ends, respectively. The introns were very AT rich, being 89.1% and 83.6% composed of AT residues, respectively, compared to the cDNA encoding the Muguga PIM which contained 61.0% AT residues. The predicted protein coding region was 57.6% composed of AT residues.

### **E2a.8 Expression of the polymorphic immunodominant molecule of *Theileria parva* in transfected COS cells**

**Scientists:** P. Toye, M. Metzelaar\*, H Clevers\*

**Technician:** J. Nyanjui

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Utrecht, The Netherlands

Because of the importance of transfection as a technique in the identification of *Theileria parva* antigens, the characteristics of expression of the polymorphic immunodominant molecule (PIM) antigen of *T. parva* in COS cells was examined.

Examination of the cDNA sequence of the *T. parva* Muguga PIM revealed a putative leader sequence at the amino end and a hydrophobic sequence at the carboxyl end suggestive of a membrane-anchor region. FACS analysis showed that live COS cells transfected with the Muguga PIM cDNA reacted with anti-PIM MAb, suggesting that the theilerial leader sequence and transmembrane regions were functional in mammalian cells. Deleted versions of the PIM expression plasmid were constructed which lacked either the leader sequence (PIM<sub>l</sub>-) or the hydrophobic carboxyl domain (PIMHis<sub>362</sub>). When transfected into COS cells, neither plasmid induced the expression of PIM on the cell surface, although the protein was detected in fixed and permeabilized cells. Thus, it appears that both the leader sequence and the carboxyl hydrophobic region are essential in transporting PIM to the surface of COS cells.

In the course of making the PIMHis<sub>362</sub> construct, another plasmid which terminated at Thr<sub>354</sub> was constructed. When this plasmid was transfected into COS cells, the PIM antigen could not be detected in fixed and permeabilized cells by anti-PIM MAb. These results suggested that the truncated protein is rapidly exported from the cell. To test this hypothesis, a version of the PIMThr<sub>354</sub> expression plasmid which also lacked a leader peptide was constructed. COS cells transfected with this plasmid were readily detected with anti-PIM MAb.

It was presumed that the lack of surface expression in COS cells transfected with the carboxyl-deleted version of PIM was due to the loss of the hydrophobic region amino acids 408 to 446. To confirm this, another version of the PIM expression plasmid, PIMAla<sub>343</sub>, was constructed. This plasmid contained the hydrophobic region but not the terminal 37 amino acids of the predicted protein. When transfected into COS cells, PIM could not be detected on the cell surface probed with MAb before fixation and permeabilization of the cells. It therefore appeared that amino acids in the terminal 37 residues were essential for expression of PIM on the surface of transfected COS cells.

The predicted protein sequences of two versions of the PIM protein from two different stocks of the parasite both ended with a pair of cysteines. This terminal Cys-Cys motif is present on all *rao* proteins and is believed to aid in targeting proteins to endosomal compartments. When a plasmid encoding all but the final two cysteines of PIM was transfected into COS cells, the antigen was readily detected on the cell surface. This result indicates that the double cysteine tail of PIM does not contribute to surface expression of PIM, and that there are amino acids between Ala<sub>443</sub> and Cys<sub>479</sub> which are necessary for the surface location of the antigen in transfected COS cells.

### **E2a.9 Inoculation of cattle with the *Theileria parva* polymorphic immunodominant molecule (PIM)**

**Scientists:** P. Toye, V. Nene, S. Morzaria, A. Musoke

**Research Associates:** P. Spooner, E. Gobright, R. Skilton

**Technicians:** J. Nyanjui, S. Wanyoni

A previous report indicates that monoclonal antibodies and polyclonal antisera to the *Theileria parva* polymorphic immunodominant molecule (PIM) neutralized the infectivity of sporozoites *in vitro*. (ILRAD 1989 Annual Scientific Report, p. 17). To

evaluate the immunizing capacity of this antigen, three experiments were conducted in which cattle were given various forms of PIM.

In the first experiment, seven cattle were inoculated with NS1.PIM emulsified in a proprietary adjuvant. NS1.PIM is a fusion protein consisting of the non-structural protein of the influenza virus and PIM. After one inoculation, all cattle developed large swellings at the site of inoculation, preventing further administration of the antigen. None of the cattle produced neutralizing antisera after the single inoculation and none was protected against challenge with sporozoites of the *T. parva* Muguga stock.

In the second experiment, cattle were inoculated with NS1.PIM or gst.PIM, the latter being a fusion protein of PIM and glutathione S-transferase. Two cattle were inoculated with NS1.PIM in saponin, two cattle were given gst.PIM in saponin and two more received gst.PIM in the proprietary adjuvant. In addition, one animal received gst fused to amino acids 3 to 75 of PIM. This region was selected as it contains the epitopes of several neutralizing MAb. No adverse reactions at the site of inoculation were observed. After five inoculations, although anti-PIM antibodies were detected by ELISA, none of the animals showed sporozoite neutralizing activity in their sera, and none was protected against a 1:75 dose of sporozoites of the *T. parva* Muguga stock.

To determine whether the failure to induce neutralizing antisera was a consequence of using a bacterially-derived fusion protein, a third experiment was conducted in which seven cattle were inoculated with a form of the PIM antigen expressed by baculovirus and seven animals received a lysate of infected bovine lymphocytes. The cattle were given a total of five inoculations each at two weekly intervals, except for the fourth inoculation which was administered one month after the third. All cattle produced antibodies to PIM, although in several cases the titre of antibody measured by ELISA declined after the fifth inoculation. None of the cattle produced neutralizing antibodies in response to inoculation with the different antigen forms and none of the cattle were protected against challenge with a 1:100 dilution of the *T. parva* Muguga stabilate.

### **E2a.10 Lack of intron splicing of theilerial genes transfected into COS cells**

**Scientists:** P. Toye, V. Nene

**Technicians:** J. Nyanjui, S. Wanyonyi

The polymorphic immunodominant molecule (PIM) gene of *Theileria parva* contains two introns of 55 bp and 61 bp respectively. To determine if they were properly spliced in COS cells, the genomic DNA encoding PIM was inserted into the same eukaryotic expression plasmid used for the cDNA [see Abstract E2a.7]. After transfection into COS cells and probing with anti-PIM MAb, reactivity was detected with only one to two cells per well plated with about  $10^5$  transfected cells, compared with about  $10^3$  to  $10^4$  per well of PIM cDNA-transfected cells. It was suspected that the small introns were not being spliced by the COS cells, and that translation was terminating at a stop codon within the first intron, with the result that the truncated protein was behaving like pThr<sub>354</sub> [see Abstract E2a.8] and being rapidly exported

from the cells. To test this, the leader sequence from the genomic plasmid was deleted and transfected into COS cells. When probed with anti-PIM MAb, cells expressing PIM were detected at a similar frequency to that for cells transfected with cDNA.

Another *T. parva* gene, that encoding the sporozoite antigen p67, has been found to contain a 29 bp intron. The cDNA and genomic versions of the gene were transfected into COS cells and the expressed proteins compared by immunoblot. The results indicated that a truncated version of p67 is present in COS cells transfected with genomic DNA. The truncated protein has a Mr of 51,000, compared to 81,000 for that expressed by the cDNA. This result supports previous observations with the PIM antigen gene showing that small theilerial introns are not efficiently removed in transfected COS cells and indicates a limitation of using genomic libraries expressed in COS cells and bacteria for identifying genes of interest.

### **E2a.11 Identification of novel sporozoite antigens of *Theileria parva* that induce neutralizing antibodies**

**Scientists:** A. Musoke, V. Nene

**Technicians:** L. Gichuru, J. Ngugi

A search for novel antigens of sporozoites of *Theileria parva*, other than p67 and PIM, which might constitute vaccine candidates was initiated. A sporozoite lysate was fractionated through an SDS-polyacrylamide gel and protein bands, revealed by Commassie blue staining, were recovered by electroelution from the gel pieces. The eluted proteins ranged in Mr from 30 to 110 kDa. Eighteen distinct eluates were obtained and each eluate was inoculated into a group of rats in Freund's adjuvant. Sera were obtained from the rats after four inoculations and checked for the protein they identified in Western blots. The results indicated that the major antigens recognized by the various sera were 30, 32, 55, 67 and 110 kDa in size. Some of the rats did not respond to the immunogens.

Sera showing antigen specificities were tested in neutralization assays. Good neutralization was observed in sera containing antibodies to p67 and p55 (a break-down product of p67). Sera containing antibodies to p30, p32 and p110 showed partial neutralization. Monoclonal antibodies against these proteins have been generated and they are being assessed for their capacity to neutralize the infectivity of sporozoites. The gene encoding p110 has been cloned.

**E2b.1 Screening of cDNA clones isolated from *Theileria parva*-infected lymphocytes with cytotoxic T lymphocytes**

**Scientists:** P. Wijngaard\*, H. Clevers\*, P. Toye

**Technician:** J. Gachanja

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The isolation and partial sequencing of 77 separate clones from a large molecular size (> 2.2 kb) cDNA library from a cell line infected with *Theileria parva* (Muguga) schizonts has been described (*ILRAD 1993 Annual Scientific Report*, p. 24). Of these clones, 36 were considered to be in the correct orientation to allow for expression in COS cells. Two assays were performed to determine if the clones encoded antigens recognized by immune CTL. Firstly, the 36 plasmids were divided into nine pools of four and each pool transfected into COS cells expressing the class I MHC molecule KN104. After two days each transfected population was assayed for recognition and lysis by a KN104-restricted CTL line.

In the second assay, transfected COS cell populations prepared as above were used to restimulate the CTL line, which was assayed for cytotoxic activity after seven days on an autologous *T. parva*-infected cell population. No consistent positive results were obtained with a plasmid pool in either assay, suggesting that the antigen(s) recognized by the CTL was not encoded by any of the isolated plasmids.

**E2b.2 Development of a TNF-based assay to detect antigens recognized by CTL from *Theileria parva* immune cattle**

**Scientists:** P. Toye, M. Sileghem, P. Wijngaard\*, V. Nene

**Technician:** J. Gachanja

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Utrecht, The Netherlands

Antigens recognized by tumour-specific CTL have been identified by assaying tumour necrosis factor (TNF) release from CTL stimulated with fractions of a cDNA library constructed from target cell mRNA and transfected into COS cells (Brichard *et al.* (1993) *Journal of Experimental Medicine* 178: 489). In these assays, TNF release was measured by lysis of WEHI-13 cells. As a first step in applying this system to the identification of antigens recognized by CTL from cattle immune to *Theileria parva* infection, the ability of bovine TNF, as released from specifically restimulated CTL, to lyse WEHI-13 cells has been determined.

The results indicated that supernatants from a CTL bulk cell line specifically restimulated with autologous infected cells induced detectable lysis of WEHI-13 cells. The lytic activity was inhibited by an anti-TNF monoclonal antibody and there was little background release from unstimulated CTL or infected cells alone.

To develop a library for use in the screening assay, a small low molecular size (< 2.2 kb) fraction of a cDNA library of the *T. parva* (Muguga) schizont-infected lymphoblastoid cell line, E98 Tpm, has been subdivided. 551 plasmid DNA preparations consisting of about 200 colonies each were made from the library and stored as DNA and glycerol stocks.

Because a cDNA library made from isolated schizonts would require the screening of fewer clones, procedures were developed to purify rapidly *T. parva* (Muguga) schizonts released from infected host cells. cDNA was made from this parasite-enriched mRNA and cloned into pCDM8 using a BstXI adaptor carrying a translational start codon. The library consists of about 250,000 colonies with insert sizes ranging from 500 bp to 3.5 kb. Two clones which hybridized to piroplasm genomic DNA were sequenced. One clone encodes *Tpr* sequences and the other is identical to a cDNA isolated by the Utrecht laboratory.

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

**Scientists:** E. Taracha, A. Musoke, D. McKeever, W. Fish, D. Hunt\*, H. Stauss†

**Research Associate:** C. Nkonge

**Technicians:** J. Gachanja, E. Awino

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A peptide sensitization assay has been developed for identifying high performance liquid chromatography (HPLC) purified peptides that contain epitopes of *Theileria parva*-specific cytotoxic T lymphocyte (CTL) clones. The assay utilized two target cell systems comprising, firstly, a cell line infected with a parasite clone that is not recognized by the *T. parva* (Muguga)-specific CTL clone (T20.40) used for screening and, secondly, using the clone itself as a target based on a fratricide effect. Although the system has allowed the detection of HPLC-purified peptides that sensitize targets to lysis by the CTL clone, it has lacked reproducibility.

Since peptides were isolated from a parasitized cell line which was not autologous to the target cells used for sensitization, it is possible that binding of the purified peptides to the restricting class I MHC elements was variable leading to inconsistent recognition of the targets by clone T20.40. Efforts have therefore focused on re-developing the sensitization assay to make it reproducible. This has involved use of peptides isolated from trifluoroacetic acid (TFA) lysates of autologous parasitized cells (approximately  $1 \times 10^{10}$ ) and resolved by reverse-phase HPLC using a TFA/acetonitrile gradient. The purified peptides were used to sensitize cloned autologous Concanavalin A-induced blasts to lysis by a polyclonal *T. parva*-specific effector line. In another approach, the cytotoxic T cell line was itself sensitized with the peptides and used as a target in a fratricide assay. Both of these approaches have demonstrated repeatable activity against target cells sensitized with five purified peptide fractions eluting in tandem on the HPLC column. However since some

fractions were toxic and caused spontaneous lysis of target cells in the absence of effector CTL, Con A blasts have been used in subsequent experiments. In a separate experiment utilizing peptides isolated from  $1 \times 10^9$  autologous parasitized cells, the positive fractions were pooled and further re-chromatographed yielding distinct peaks. The subfractions were subsequently tested for their ability to sensitize targets for lysis by the bulk effector line, and this has shown two distinct peaks of activity. CTL clones generated from the polyclonal effector line will be used to screen the positive fractions re-resolved on two different gradients. Peptides in the two sets of samples recognized by CTL will be sequenced by micro-sequencing techniques.

### **E2c.1 Molecular cloning, expression and characterization of a candidate vaccine antigen of *Theileria mutans***

**Research Associates:** R. Skilton, J. Katende, C. Wells, P. Spooner  
**Scientists:** S. Morzaria, A. Musoke, V. Nene

Cattle that have recovered from natural infection with *Theileria mutans* or have been immunized with semipurified lysates of piroplasms are immune to challenge. In such cattle the predominant antibody response is to the 32 kDa antigen (p32). This antigen has been localized on the surface of *T. mutans* piroplasms. It is possible that the antigen may be involved in inducing a protective immune response. The gene for the 32 kDa piroplasm antigen has been cloned by PCR of cDNA using a primer based on the N-terminal sequence of purified antigen and three cDNA variants, clones 9, 21 and 23, have been sequenced. Full length cDNA inserts from clones 21 and 23 were cloned into pGEX-11T and pMG1 bacterial expression vectors giving fusions with glutathione S-transferase (GST) and the non-structural protein of influenza virus (NS1), respectively. GST fusions gave good yields of pure recombinant p32 (up to 6 mg/litre bacterial culture). NS1 fusion protein proved difficult to purify and yields were poor (NS1/p32 could only be detected by Western blotting). GST fusion proteins were therefore used for subsequent work.

Two cattle were immunized and boosted once with GST/clone 23. Two weeks after the boost both animals had titres of antibodies against GST/clone 23 in excess of 1:500,000. Western blotting with lysates of *T. mutans* piroplasms showed these antisera to recognize native p32. The production of high titre antibodies that recognize native p32 indicates GST/clone 23 may be a candidate vaccine antigen. As an alternative system for producing recombinant p32, clone 23 cDNA was subcloned into the baculoviral transfer vector pACL11. Plaques of insect cells harbouring recombinant baculovirus expressing p32 were identified by Western blotting. One such plaque, clone 1, has been expanded in culture for further work to determine its suitability as a source of recombinant p32.

In order to confirm that the sequence polymorphism detected in the original clones (clones 9, 21 and 23) was not due to artifacts introduced during PCR through the low fidelity of *Taq* DNA polymerase, it was decided to sequence genomic clones encoding the p32. Partial sequences from six genomic clones derived from a  $\lambda$ gt11 *T. mutans* library indicate polymorphisms within the p32 sequences and also within flanking sequences. These results are consistent with the polymorphisms seen within cDNA sequences for p32. Previous data from Southern blotting indicated that the p32 gene is a single copy. Since the source of the material for the gDNA and p32 cDNA clones was a single, uncloned isolate, genomic sequence polymorphisms therefore confirm that a single population of *T. mutans* is highly polymorphic. The initial sequencing data also show, for each of the clones, the position of the initiation codon and the presence of a signal sequence. A signal sequence is consistent with the localization of p32 on the piroplasm surface and also with the finding of signal sequences in homologous antigens of *T. sergenti* and *T. buffeli*.

**E2c.2 Identification of bovine B-cell epitopes on the immunodominant 32 kDa antigen (p32) of *Theileria mutans*****Research Associate:** R. Skilton**Scientists:** S. Morzaria, A. Musoke

Studies have indicated that one of two full length cDNA inserts (clone 23) of *Theileria mutans* expressed as a fusion with GST in the pGEX bacterial expression system may be a suitable marker for the diagnosis of *T. mutans* infection of cattle in an antibody detection ELISA. Since there is sequence polymorphism within p32, several factors remaining to be determined before the assay can be used for wide scale epidemiological or diagnostic application include identification of the epitopes recognized by bovine B-cells, conservation of epitopes within the polymorphic p32 from a single isolate and determination of whether the epitopes in the GST/clone 23 are conserved within isolates of *T. mutans* from across sub-Saharan Africa.

For the identification of B-cell epitopes, libraries were constructed in the phage expression vector  $\lambda$ gt11 with random 50–150 bp fragments of p32 cDNA clones 9, 21 and 23. Approximately 15,000 pfu of each amplified library were immunoscreened with bovine serum BL-99 (bovine anti-GST/clone 23). Inserts of immunopositive plaques were amplified by PCR and sequenced directly by a PCR cycle sequencing system.

Two epitopes have been mapped. The first, epitope number 1, is present in clones 9, 21 and 23, maps to amino acids 8–15 of p32, and has the sequence: EPKDLTVN. The second, epitope number 2, is present in clones 9 and 23 only, maps to amino acids 212–229 and has the sequence QTEANKLLHAMDSNWPAD. Clone 21, which fails to express epitope number 2, has the following sequence for amino acids 212–229: QTEANKLLHAMDATWPLD (amino acid differences are underlined). Epitope number 2 may reside in the region of these amino acid differences in clones 9 and 23. Epitope mapping of epitopes reactive with field sera and bovine sera experimentally raised against *T. mutans* piroplasm lysates is in progress.

**E2c.3 Identification and characterization of the gene encoding the 200 kDa polypeptide of *Babesia bigemina*****Research Fellow:** N. Tebele**Supervisors:** A. Musoke, S. Morzaria, V. Nene**Research Associates:** R. Skilton, J. Katende

The 200 kDa antigen of *Babesia bigemina* has been identified as a candidate antigen for use in an antibody detection ELISA. The recombinant antigen produced as a fusion protein with GST in *Escherichia coli* has been used in an antibody detection ELISA to determine its specificity [see Abstract E1b.4]. Bovine sera from an area where *B. bigemina* is absent have been found to be cross-reactive with the antigen. This has prompted studies to define epitopes of the *B. bigemina* antigen recognized by hyperimmune sera. Epitope libraries of DNase digested fragments of the 3.8 kb

insert in pUC 18 were constructed in  $\lambda$ gt11. The libraries containing insert sizes ranging from 100–500 bp were screened with bovine hyperimmune sera. Of the many positive clones, the one that gave the strongest signal (construct 1) was cloned into pGEX and expressed a fusion protein with GST of 33 kDa. Construct 1 was sequenced and found to have an open reading frame of 190 bp. The fusion protein was not recognized in an antibody ELISA by bovine antisera derived from animals with experimentally infected *B. bovis*, *Anaplasma marginale*, *Theileria parva*, *T. mutans*, *T. buffeli*, *Cowdria ruminantium* or *Trypanosoma congolense* experimentally infected animals. However the antigen was not recognized by a panel of 12 monoclonal antibodies (MABs) raised against the native 200 kDa polypeptide. A second construct (construct 2) recognized by both the MABs and recovered bovine sera was expressed and characterized in an ELISA. The antigen was found to react very weakly with *B. bigemina* hyperimmune sera but reacted very strongly with the panel of 12 MABs. This result indicated that construct 2 is unsuitable for producing a diagnostic antigen but useful for mapping the MAB-reactive epitopes on this protein. Construct 1 is currently being characterized further using a panel of defined experimental and field sera.

### **E3a.1 Mechanisms of protection in cattle that resist challenge with *Theileria parva* after immunization with NS1-p67**

**Scientists:** A. Musoke, D. McKeever, E. Taracha

**Research Associates:** C. Nkonge, J. Katende

**Technicians:** E. Awino, J. Gachanja

Previous experiments have shown that 70% of cattle immunized with the NS1-p67 recombinant form of the *Theileria parva* major sporozoite surface antigen resist challenge with the parasite. The degree of protection in immunized animals is not associated with antibody titre, isotype specificity, affinity or peptide specificity. It has been reported that p67 remains on the surface of the recently infected cell after sporozoite invasion and this observation highlights the possibility that the antigen might be taken up by the cell and expressed on its surface in association with class II MHC molecules. Thus a possible protective mechanism is the activation of CD4<sup>+</sup> T cells that might exert parasiticidal or parasitastatic effects in the lymph node that drains the site of challenge within the draining lymph.

This question was addressed in a group of seven cattle immunized with NS1-p67 that had shown no reaction to sporozoite challenge by rechallenging them with recently infected autologous lymphocytes. These cells were prepared by incubating peripheral blood mononuclear cells with an excess of sporozoites for 30 min and eliminating residual sporozoites by complement lysis in the presence of a p67-specific MAb. Animals received  $1 \times 10^6$  recently infected cells, an inoculum calculated to approximate the infective dose contained in one infected acinus of a tick salivary gland.

All animals developed severe reactions to challenge with a 70% lethal dose of the homologous sporozoite stock, suggesting that their immunity did not incorporate a

significant component directed at the infected cell. These observations support a major role for serum antibody in the protection of NS1-p67-immunized cattle against challenge, but do not provide an explanation for the lack of correlation between protection and those serological parameters that have been measured in immunized animals. Additional experiments are now in progress that involve the transfer of CD4<sup>+</sup> T cells between NS1-p67-immunized and naive twin calves.

### E3b.1 Attenuated *Salmonella* strains expressing *Theileria* antigens

**Scientist:** V. Heussler

**Technician:** W. Mwangi

A number of different expression constructs for expression of the p67 *Theileria parva* sporozoite antigen have been made and tested in *Salmonella dublin* strain SL5631. The p67 plasmid constructs that were already available at ILRAD (pGEX67, pMC622) showed good expression of the antigen in SL5631, but were not stable in the bacteria *in vitro* or *in vivo*. The p67 gene was therefore subcloned in the pJFF224XN plasmid vector, which is stable in *Salmonella*. However, immunoblot analysis of transformed *Salmonella* revealed weak expression of the p67 antigen. To enhance expression, a new construct was made incorporating the *tac* promoter upstream of the p67 gene. This construct, designated pJV67p, gives rise to good levels of expression of p67 and was used for further studies.

To test the stability of *Salmonella* p67 constructs *in vivo*, mice were infected orally with SL5631 transformed with pGEX67, pMC622 or pJV67p. Control mice were infected with the untransformed stock. Mice were sacrificed on days 1 and 3 and the liver, spleen and small intestine were removed. Bacteria were detected on day 1 after infection but were undetectable by day 3.

In a separate experiment, mice were infected intraperitoneally with the same SL5631 p67 constructs so that the immune response to the antigen could be monitored. Serum was taken at weekly intervals and assayed for antibodies to p67 and *Salmonella* antigens as a control. A high titre of anti-*Salmonella* antibodies could be detected, but a response to the recombinant p67 was not observed. T cell proliferation assays performed with the spleen cells of the infected mice showed similar results; the cells responded well to the crude *Salmonella* antigen, but not to the p67 antigen.

These observations suggest that, at least for mouse immunizations, strain SL5631 is not an effective delivery vehicle for p67. The p67 constructs were therefore used for transformation of *Salmonella typhimurium* strain SL3261 and tested for expression in immunoblotting assays. All constructs showed a much lower expression level in SL3261 than in SL5631. In particular, the pJV67p plasmid expressed the antigen only in small amounts. A new construct, pJV67p+, was made that incorporates an amino acid change between the phosphatase secretion signal and the p67 antigen. This modification gives rise to a 10 fold increase in the expression of the p67 antigen.

Preliminary experiments in mice revealed that the SL3261 strain infects liver and spleen very efficiently and persists for at least 10 days. Other mice have been infected with SL3261 expressing p67 from the pGEX67, pMC622 and pJV67p+ plasmids to determine if p67-specific immune responses are generated after inoculation.

**E3c.1 Inductive requirements of bovine cytotoxic T lymphocytes (CTL) specific for defined antigens of *Theileria parva*****Scientists:** E. Taracha, D. McKeever**Technicians:** W. Mwangi, F. Mbwika, E. Awino

Previous observations have shown that the generation of bovine CD8<sup>+</sup> CTL recognizing *Theileria parva*-infected lymphoblasts requires input from antigen-specific CD4<sup>+</sup> T cells. Experiments were conducted to examine factors important for the induction of CD8<sup>+</sup> CTL to *T. parva*-defined antigens presented by various antigen presenting cells (APC). Initially, this work has focused on the p104 rhoptry antigen cloned in the p1114 shuttle vector of vaccinia virus (vv) which was used to generate vv recombinants (VVp104) expressing the antigen. Two calves were inoculated intradermally with  $5 \times 10^8$  pfu of VVp104 and, after three weeks, assays were performed to determine the reactivity of T cells in peripheral blood mononuclear cells (PBMC) from the two calves. In preliminary experiments with PBMC cultured in the presence of graded amounts of sporozoite lysate or heat-killed sporozoites, it was demonstrated that p104-reactive CD4<sup>+</sup> T cells were generated in cultures of immune but not naive PBMC. Separate experiments to determine the presence of CD8<sup>+</sup> T cells recognizing p104 have utilized autologous peripheral blood monocytes and skin fibroblasts as APC in the generation and assay of antigen-specific CTL. Co-cultures of PBMC with glutaraldehyde-fixed VVp104-infected monocytes, in the presence of 5% supernatant of T cell growth factors, generated effector cells which recognized and lysed skin fibroblasts infected with VVp104 or wild type vv. This cytotoxic activity, which was class I MHC restricted and CD8<sup>+</sup> T cell-mediated, was approximately 1.5-fold higher on VVp104-infected targets than on vv-infected targets. This suggests that CD8<sup>+</sup> T cells responding to p104 antigen occur in PBMC from the two calves following immunization. Further critical experiments to establish the presence of p104-specific CD8<sup>+</sup> T cells in VVp104-immunized calves will utilize T cells or skin fibroblasts transfected with the p104 gene as APC. Currently, the p104 gene has been cloned from pUC19 into the pCDNA3neo plasmid and sequenced prior to transfection. With the availability of p104 transfectants, studies on the factors required to induce p104-specific CD8<sup>+</sup> CTL are planned utilizing sorted T cells from immunized calves. p104-specific CTL clones will be generated and used in antigen presentation assays.

**E3c.2 Analysis of cytokine expression by *Theileria parva*-transformed lymphocyte populations****Scientists:** D. McKeever, E. Taracha**Research Associate:** N. MacHugh**Technicians:** A. Kaushal, E. Awino

Recent observations in murine systems of apparently identical T lymphocyte subsets with distinct profiles of cytokine expression have highlighted the need for practical

methods for detecting cytokines in bovine immune cell populations. A convenient technique for analysing cytokine expression in cells after stimulation *in vivo* or *in vitro* is PCR amplification of first strand cDNA prepared from the cells, using cytokine-specific primers. A set of primers have been designed for amplifying bovine IL-2, IL-4, IL-10 and the  $\alpha$  chain of the bovine IL-2 receptor. Additional primers for bovine IFN $\gamma$ , IL-6, IL-1 $\alpha$  were synthesized according to sequences supplied by Dr. Albert Bensaïd of CIRAD-EMVT. We have tested these primers in a preliminary analysis of the range of cytokines expressed by *Theileria parva*-infected lymphoblasts. Primers for glycero-3-phosphate were incorporated in each set of reactions to provide a standard product for comparison of level of expression.

Four bulk *T. parva*-infected lines were examined for the expression of IL-1 $\alpha$ , IL-2, IL-4, IL-6, IL-10 and IFN $\gamma$ . IL-10 and IFN $\gamma$  were expressed in all these lines whereas IL-1 $\alpha$ , IL-2, IL-4 and IL-6 were variably expressed. To address the possibility that sub-populations having distinct cytokine profiles were present within these lines, additional experiments were carried out using two infected B-cell lines and infected lines of CD4<sup>+</sup>, CD8<sup>+</sup> and  $\gamma\delta$  T cell lineages derived from two separate animals. Only IL-10 was expressed universally by these lines. The two B-cell lines expressed only IL-6 in addition, while lines of CD4<sup>+</sup>, CD8<sup>+</sup> and  $\gamma\delta$  phenotypes showed variable expression of IL-1 $\alpha$ , IL-6 and IFN $\gamma$ . IL-2 was not expressed by any of the lineage-defined lines.

These results indicate that infection of lymphocytes with *T. parva* does not impart a specific cytokine profile, suggesting that the range of cytokines expressed by infected cells may reflect the activation state of the cell prior to infection. However, this study did not examine synchronized cultures, and the possibility cannot be excluded that cytokine expression varies with the stage of the growth cycle. A consistent finding with all of the cell lines was the expression of IL-10. This cytokine is associated in murine systems with the TH2 subset of CD4<sup>+</sup> lymphocytes, and is known to regulate the activity of the TH1 subset. Differential responses in these subsets have been shown to influence the outcome of several infectious diseases. The possibility that expression of IL-10 by *T. parva*-infected cells is associated with the considerable immunopathology that characterizes primary infection with the parasite will be investigated in collaboration with the Institute for Animal Health, Compton, UK.

### **E3c.3 Activation of bovine $\gamma\delta$ T cells by *Theileria parva*-transformed cell lines**

**Scientists:** C. Daubenberger, D. McKeever

**Research Associate:** L. Gaidulis

**Technician:** D. Ngugi

T cells bearing the gamma/delta antigen receptor ( $\gamma\delta$  T cells) constitute up to 50% of T cells in peripheral blood and lymphoid organs of calves below six months of age. So far, little is known about their involvement in immune responses in general and against *Theileria parva* in particular.

In earlier work it was established that bovine  $\gamma\delta$  T cells proliferate *in vitro* in the presence of autologous *T. parva*-transformed cell lines (TpM) and heat stressed Con A blasts. To determine the basis for this proliferative response, a panel of  $\gamma\delta$  T cell clones has been generated. These clones express the  $\gamma\delta$  T cell receptor and WC1, CD3 and CD2, but do not express CD4 or CD8.

To exclude the possibility that proliferation of the clones is an interleukin-driven effect, TpM were fixed with glutaraldehyde and used as antigen presenting cells. This treatment does not abolish proliferation of several established  $\gamma\delta$  T cell clones and lines.

Experiments with monoclonal antibodies (MAb) specific for different surface molecules located on TpM and  $\gamma\delta$  T cells revealed that the response of the clones can be blocked by the addition of GB21A, a MAb specific for the  $\gamma\delta$  T cell receptor. No blocking was observed in the presence of MAbs against MHC class I or class II molecules and CD4 or CD8.

To determine whether a known restriction element is used by the  $\gamma\delta$  T cell clones, Con A blasts and TpM were established from a variety of MHC-mismatched animals and used as stimulator cells in co-cultivation assays. None of the Con A blast lines was capable of stimulating the clones whereas all the TpM established from these animals induced proliferative responses. This suggests that either parasite-induced or parasite-derived antigens are responsible for the activity of the clones. To confirm this, TpM were cured of the parasite using buparvaquone. TpM lost their stimulatory capacity for  $\gamma\delta$  T cell clones after five days culture in the presence of the drug.

Antigenic heterogeneity is a characteristic of *T. parva* parasite stocks. To establish whether  $\gamma\delta$  T cells react with conserved or polymorphic antigens, a CD4<sup>+</sup> Con A-induced  $\alpha\beta$  T cell clone was infected with a number of different parasite stocks. Ten different cell lines stimulated  $\gamma\delta$  T cells in a concentration-dependent manner, although with different intensities.

Cytokine profiles of the clones have been examined using reverse transcription-PCR (RT-PCR) after 14 days stimulation by TpM. Preliminary results show that the cells produce INF $\gamma$ , TNF $\alpha$ , IL-4 and the IL-2 receptor  $\alpha$  chain but that message for IL-2, IL-6 and TNF $\alpha$  could not be detected.

#### **E3c.4 Identification, purification and partial characterization of hsp 70 kDa proteins expressed in *Theileria parva*-transformed cells**

**Scientists:** C. Daubenberger, N. Tsuji\*, D. Nandan, D. McKeever

**Research Associate:** E. Gobright

**Technicians:** J. Osaso, R. Thatthi

\*National Institute of Animal Health  
Tsukuba, Japan

Preliminary results suggested that a proportion of bovine  $\gamma\delta$  T cells are activated by *Theileria parva*-transformed cell lines (TpM) and by heat-stressed Concanavalin A

induced blasts (Con A blasts). Several reports in the literature show that a proportion of  $\gamma\delta$  T cells in mouse and human can be activated by stress proteins of the 60 and 70 kDa families. The possibility that bovine  $\gamma\delta$  T cells, activated by infected cell lines, recognize stress proteins has been examined by characterizing the expression of heat shock proteins (hsp) of 60 and 70 kDa.

Con A blasts and TpM were exposed to heat stress at 42 °C for varying periods. Cells were metabolically labelled and analysed by non-equilibrium pH gradient gel electrophoresis (NEPHGE) in the first dimension and SDS-PAGE in the second. It was observed that Con A blasts express two different inducible members of the hsp 70 kDa family while TpM express three. The kinetics of expression of the hsp 70 kDa proteins does not differ between Con A blasts and TpM. No expression of members of the 60 kDa families could be detected. In addition to 70 kDa hsp proteins, a single 90 kDa hsp protein was induced in Con A blasts and TpM.

To examine the constitutive expression of hsp 70 kDa proteins, non-labelled cells were analysed by NEPHGE and SDS-PAGE. Preliminary results indicated that TpM constitutively express two hsp 70 proteins. Western Blot analysis revealed that these species differ immunologically, because only one is recognized by MAb SPA-820, a commercial reagent specific for hsp 70. We have now generated an additional MAb against isolated hsp 70 kDa proteins derived from TpM. In immunofluorescence analysis of TpM this MAb binds only to schizonts and it does not recognize Con A blast proteins.

To develop a transfection system in which hsp 70 kDa proteins can be evaluated as targets for parasite-specific  $\gamma\delta$  T cells, two cDNA clones isolated from a TpM expression library (by Dr. P. Wijngaard, Utrecht, Holland) have been partially sequenced to confirm that they encode hsp 70 proteins. These clones are currently being cloned into eukaryotic expression vectors for transfection.

### **E3c.5 Analysis of the basis of protection in cattle immunized with killed *Cowdria ruminantium* (Gardel) in complete Freund's adjuvant**

**Scientists:** P. Totte\*, D. McKeever, A. Bensaid\*

**Technician:** J. Mburu

\*CIRAD-EMVT, Maisons-Alfort, France

Infectivity of the Gardel strain of *Cowdria ruminantium* for cattle has been confirmed. Attenuation of this strain for cattle after 86 passages (approximately ten days per passage) in endothelial cells *in vitro* was also confirmed. Animals infected with attenuated *C. ruminantium* were fully protected against challenge with an earlier, virulent passage of the pathogen.

A group of five dairy cattle was immunized by intramuscular inoculation of 0.5 ml of *C. ruminantium* lysate formulated in an equal volume of Freund's complete adjuvant followed one month later by a similar inoculation formulated in Freund's incomplete adjuvant. PBM cells collected from all these animals fortnightly after primary inoculation were shown to proliferate *in vitro* in response to *Cowdria*

antigens. FACS analysis revealed that the majority of proliferating cells are CD4<sup>+</sup> and express the IL-2 receptor. Cloning of these *Cowdria*-responding T cells is under way.

Two of these animals were challenged two months after boosting with live virulent *C. ruminantium* and were fully resistant to infection. The remaining animals have yet to be challenged. Although FACS analysis of PBM cells collected at intervals after challenge revealed a significant increase in the proportion of monocytes and CD8<sup>+</sup> T cells, similar changes were observed in the challenge control animal which succumbed to the disease.

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# TRYPANOSOMIASIS

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**T1a.1 Sampling designs to estimate the prevalence of cattle trypanosomiasis: a prevalence study in Northern Kwazulu, Republic of South Africa****Scientists:** P. Lessard, R. Masake, D. de Waal\*, R. Carter†**Research Associate:** S. Minja

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†Department of Agriculture, Kwazulu Government, Republic of South Africa

The most common type of epidemiological study of cattle trypanosomiasis performed in Africa is the prevalence survey. Such studies are used to determine the level of disease in a population at a specific point in time, and to evaluate the effects of vector and/or parasite control programmes. The sampling design of such prevalence surveys depends strongly on the number of groups of cattle that are available for sampling and the correlation of disease factors within these groups. Groups of cattle that are commonly available for sampling include animals associated with administrative areas, dip tanks, villages and herds. Knowledge of the correlation of trypanosome infections, as detected by species-specific antigen-ELISA test and/or parasitological techniques, within these sampling groups would help to determine the appropriate number of areas, dip tanks, villages, herds and animals to sample. The objective of this study was to calculate the level of correlation of cattle trypanosomiasis within large sampling groups in order to improve the design of prevalence surveys.

The study used a prevalence survey of cattle trypanosomiasis conducted early in 1994, in Northern Kwazulu, Republic of South Africa. The prevalence estimates were calculated using the buffy coat technique (BCT) and the antigen-ELISA test. The cattle population was 313,624 cattle in three districts. Each district was divided into sections and one or more sections were assigned to a dip tank. There were 196 sections and 106 dip tanks. If a dip tank serviced more than one section, cattle from each section visited the dip tank on different days of the week. A two-stage sampling design was used for this study. The primary unit, or first stage, was the section. A list frame of all sections in each district was compiled. Sections were randomly selected, with probability proportional to the number of cattle they contained. The sample size determination of sections was based on the percentage of sections in each district. The secondary unit, or second stage, was cattle. Cattle were selected systematically as they moved through the dip tank. The sample size of cattle was based on a simple random sampling design for each district. The estimated prevalence was 50% with a required precision of 5% for a level of confidence of 95%.

Based on these parameters the calculated sample size was 383 cattle per district. Using an intra-section correlation coefficient of 0.2, the adjusted sample size per district was calculated to be 703 cattle. However, in order to calculate a more accurate correlation coefficient within these groups, the sample size was increased to a total of 4589 animals in three districts. Serological and parasitological prevalence estimates are currently being calculated. Intra-section, intra-dip tank, intra-herd, intra-district and intra-ecological zone correlation coefficients will be determined. Based on these correlation coefficients within sampling groups, a corrected sample size and sampling design will be performed on the same data set and the estimate of prevalence with 95% confidence intervals compared to the estimate of prevalence with 95%

confidence intervals originally calculated. A benefit-cost analysis will also be performed to evaluate the financial gain associated with the various sampling designs.

**T1a.2 Use of PCR and DNA probes to confirm the presence of trypanosome infections in cattle from an area of Northern Zanzibar thought tsetse free.**

**Scientists:** P. Lessard, R. Masake, P. Majiwa, W. Holland\*, H. Mbwambo†

**Research Associate:** S. Minja

**Technician:** J. Njuguna

\*FAO, project URT/91/006, Zanzibar

†Animal Diseases Research Institute (ADRI), Tanzania

Tsetse fly eradication has been chosen as the goal of trypanosomiasis control on the island of Unguja, Zanzibar. This decision was based on the epidemiological and ecological conditions of the island. The island is protected by a 30 km natural barrier, and only one tsetse species, *Glossina austeni*, is known to be responsible for trypanosome transmission. Although tsetse fly eradication is the objective, the programme attacks the problem from two sides: vector and parasite control. Vector control includes (i) the application of synthetic pyrethroid insecticides on living targets (livestock) via pour-on formulations or dip tanks, (ii) the use of insecticide impregnated screens and (iii) the help of the sterile insect technique. Parasite control includes (i) the treatment of all infected animals with Berenil and (ii) the control and treatment of imported livestock from the mainland.

The eradication programme is carried out exclusively in the southern two thirds of the island. The northern third of the island is not included in the programme because *G. austeni* has never been detected, clinical cases have never been recorded, and no prior survey has found the parasite in cattle of the region using parasitological techniques. However, using an antigen detection ELISA test on sera (80 samples) collected from cattle in the northern region, 44 (55%) were positive, using a cut-off point of 0.05 optical density value, to at least one of the three pathogenic trypanosomes. Due to these conflicting results between serological and parasitological tests, an investigation was initiated to confirm or refute the presence of trypanosome antigens in cattle of the area. The polymerase chain reaction (PCR) technique, combined with species- or strain-specific DNA probes, was applied to 26 blood samples collected in EDTA. DNA sequences unique to trypanosomes were amplified and hybridized in 17 (65%) samples; 8 (30%) to savannah-type *T. congolense*, 12 (46%) to a *T. vivax* probe and 3 to both. All samples were negative to Tsavo and Kilifi types of *T. congolense* and to *Trypanozoon* subgenus-specific probes. Comparison between PCR/DNA hybridization and Ag-ELISA is not currently possible due to the limited number of probes available and the unknown, but potentially high, number of trypanosome types in the field. However, 57% of the samples with *T. congolense* DNA were detected positive by the Ag-ELISA test, and 12.5% with *T. vivax* DNA were identified by Ag-ELISA.

These results suggest that trypanosome infections are present in the cattle population of the northern third of the island despite the apparent absence of tsetse

Other factors that may contribute to the high prevalence of trypanosome infections in the area are mechanical transmission by biting flies and cattle movement. Therefore, it would be extremely important to establish if tsetse-transmitted trypanosome infections occur in order to include the area in the eradication programme.

### ra.3 African Trypanotolerant Livestock Network (ATLN): tsetse component

Research Associate: S. Leak

Scientists: W. Mulatu\*, J. Rowlands†

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Studies at the ATLN sites in the Ghibe valley, southwest Ethiopia, between 1989 and 1990, demonstrated the occurrence in trypanosomes of a high level of resistance to all available trypanocidal drugs. As a result, the prevalence of trypanosomiasis in cattle rose annually from 1986 to December 1990. In January 1991 a trial was started using Cypermethrin-high-*cis* as a 'pour-on' insecticide to control *Glossina palpalipes*, *G. fuscipes* and *G. morsitans submorsitans* at the Tolley/Gullele site in the Ghibe valley. This study was initiated in order to determine the effectiveness of this tsetse control method in reducing the trypanosome prevalence in cattle in which trypanocidal drug resistance was a serious constraint. Preliminary results were given in the *ILRAD 1993 Annual Scientific Report*.

From the beginning of the experiment, between 2000 and 3000 cattle were treated at monthly intervals with a synthetic pyrethroid (RS-alpha-cyano-3-phenoxybenzyl (IRS)-*cis*, trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate) at a dosage of approximately 1 ml per 10 kg bodyweight. Treatment was given as a pour-on application along the backline, using an automatic drench gun applicator. Ovarian ageing of tsetse flies at Ghibe was used to provide data that was used to estimate the added mortality imposed on the tsetse population by this control method. By September 1994 the tsetse population was reduced by more than 93% compared with the level prior to the administration of the pour-on. Furthermore, the trypanosome prevalence in cattle as detected by the buffy coat technique was reduced by 74%, despite a high level of resistance to all available trypanocidal drugs. A significant reduction of 88% in numbers of biting flies also occurred. Whilst it is known that pour-on insecticides will control biting and nuisance flies, such a reduction has not previously been reported as a consequence of tsetse-control activities.

In association with a project to investigate the environmental impact of tsetse control, a trial was started in 1994 to control tsetse flies at a second site in the Ghibe valley, 25 km downstream from Tolley/Gullele, using a deltamethrin pour-on insecticide. A significant invasion of this part of the Ghibe valley by *Glossina m. submorsitans* took place between 1990 and 1994 and, in conjunction with the drug-resistance problem, led to a large increase in trypanosome prevalence in cattle in the area. In contrast to the study with cypermethrin, the deltamethrin insecticide

has not yet brought about a reduction in the tsetse populations. Investigations are therefore under way to determine why this treatment has not been effective.\

#### **T1a.4 A comparison of the susceptibility of allopatric populations of *Glossina pallidipes* in Kenya for stocks of *Trypanosoma vivax***

**Scientist:** S. Molloo

**Technicians:** I. Okumu, J. Muia

A colony of *Glossina pallidipes* which originated from Nguruman, Rift Valley Province, Kenya, appeared to be more susceptible to infection with a stock of *Trypanosoma vivax* (77.0%) isolated in Galana, Kenya, than a colony of *G. pallidipes* which originated from Shimba Hills, Coast Province, Kenya (71.3%). However, the difference was not statistically significant ( $p > 0.05$ ). In additional work, the *G. pallidipes* colony of Nguruman origin was significantly more susceptible to infection (80.0%) with *T. vivax* isolated in Zaria, Nigeria, than the colony of Shimba Hills origin (74.5%). Since infection rates by the two stocks of *T. vivax* in the two allopatric populations of *G. pallidipes* were markedly high, and ranged from 71.3 to 80.0%, this suggests that the vector aspects of the epidemiology of trypanosomiasis due to *T. vivax* probably do not differ between these two areas of Kenya.

#### **T1a.5 A comparison of the susceptibility of *Glossina pallidipes* originating from allopatric populations in Kenya for stocks of *Trypanosoma brucei brucei***

**Scientist:** S. Molloo

**Technicians:** I. Okumu, J. Muia

A colony of *Glossina pallidipes* which originated from Nguruman, Rift Valley Province, Kenya, was significantly ( $p < 0.05$ ) more susceptible to infection (4.4%) with a stock of *Trypanosoma brucei brucei* isolated from *G. pallidipes* in Lambwe Valley, Kenya, than was a colony of *G. pallidipes* which originated from Shimba Hills, Coast Province, Kenya (1.7%). Male *G. pallidipes* from Nguruman were significantly ( $p < 0.05$ ) more susceptible than female tsetse to this stock of *T. b. brucei*, whilst the susceptibility of sexes of *G. pallidipes* from Shimba Hills did not differ significantly ( $p > 0.05$ ). All six goats on which six infected *G. pallidipes* fed singly (three tsetse per colony), became infected. Similarly, the *G. pallidipes* colony of Nguruman origin was significantly ( $p < 0.05$ ) more susceptible to infection (6.1%) with a stock of *T. b. brucei* isolated from *G. swynnertoni* in Serengeti, Tanzania, than the colony of Shimba Hills origin (2.6%). Male *G. pallidipes* from both colonies were significantly ( $p < 0.05$ ) more susceptible than female tsetse to this stock of *T. b. brucei*. Again, all six goats on which six infected *G. pallidipes* fed singly (three tsetse per colony) became infected. If the observed differences in susceptibility of the two *G. pallidipes* colonies reflect transmission of trypanosomes by the two allopatric populations of tsetse in the field, then the epidemiology of *brucei*-trypanosomiasis must differ between these two areas of Kenya.

**A comparison of *Glossina morsitans centralis* originating from  
Tanzania and Zambia, with respect to vectorial competence for pathogenic  
*Trypanosoma* species, genetic variation and inter-colony fertility**

**Scientists:** S. Molloo, R. Gooding\*  
**Technicians:** I. Okumu, B. Rolseth\*

Department of Entomology, University of Alberta  
Edmonton, Canada

Two laboratory strains of *Glossina morsitans centralis* originating from different fly-belts (one from Singida, Tanzania, and the other from Mumbwa, Zambia) were compared with respect to vectorial competence for pathogenic *Trypanosoma* species, genetic variation and inter-colony fertility. The vectorial competence of the two colonies of *G. m. centralis* for *Trypanosoma vivax* and *T. congolense* was the same, whilst for *T. brucei brucei* it was higher in the colony of Zambian origin. Nevertheless, these two laboratory strains of *G. m. centralis* showed levels of susceptibility to the three pathogenic *Trypanosoma* species which were much greater than previously observed in laboratory colonies of other *Glossina* species. Electrophoresis of 15 enzymes revealed that the two colonies differ significantly in allele frequencies only at three loci that are relatively close together on one of the autosomes. Hybridization experiments revealed that *G. m. centralis* from the two fly-belts are consubspecific.

**T1a.7 Upsurge of the tsetse fly *Glossina swynnertoni* at Nguruman, Kenya**

**Scientists:** J. Stiles\*, L. Otieno\*, M. Chaudhury\*, S. Molloo  
**Technicians:** F. Mpanga\*, N. Darji\*

\*International Center of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya

*Glossina swynnertoni* has been reported to have a very limited distribution in northern Tanzania and southern Kenya between latitude 0° and 5° South and longitude 30° and 40° East. The altitude range of *G. swynnertoni* has also been reported to be 900–1800 m above sea level (a.s.l.). In Kenya, this tsetse species has been found previously only in the Maasai Mara game reserve, located on the western side of the Rift Valley (1.5°S, 35°E), in association with *G. pallidipes*. During routine sampling of *G. pallidipes* using NG2G traps on the Nguruman escarpment, 1600–2000 m a.s.l. (1°50'S, 36°05'E), in Kajiado District, Kenya, it was observed that, among collections totalling about 1000 flies, up to 4% were *G. swynnertoni*; the remainder were *G. pallidipes*. Similar proportions were found in tsetse populations from some of the gorges traversing the eastern side of the escarpment and contiguous regions of the Rift Valley floor, 600–700 m a.s.l. *Glossina swynnertoni* at Nguruman may have been an influx from the adjoining population in Tanzania. Alternatively, it may be part of the Maasai Mara population extending eastwards for about 50 km to the Nguruman escarpment and delimited by the Rift Valley.

**T1a.8 The saliva of tsetse flies has components that are antigenic in man and domestic animals**

**Scientists:** T. Asonganyi\*, S. Molloo

**Technicians:** J. Kabata, J. Muia

\*Faculty of Medicine and Biomedical Sciences, University of Yaoundé, Camer

Enzyme linked immunosorbent assay (ELISA) and immunoblot analyses of sera revealed that domestic animals (sheep, goats, pigs and dogs) and man exposed to feeding by tsetse flies develop antibodies to tsetse salivary antigens. High levels of anti-saliva IgG were shown in sheep, goats, pigs and dogs; IgE and IgM were not studied since anti-IgE and IgM were not commercially available for these animals. In man, the anti-saliva antibodies included IgG, IgM and IgE isotypes, indicating that some of the salivary antigens are allergenic. Antibodies in sera of people exposed to blackflies cross-reacted with saliva of tsetse, indicating that saliva of these two insect vectors have cross-reacting antigens.

**T1a.9 Trypanosomiasis in the black rhinoceros (*Diceros bicornis* Linnaeus, 1758)**

**Scientists:** S. Mihok\*, R. Olubayo†, S. Molloo

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†Kenya Agricultural Research Institute (KARI), Nairobi, Kenya

A black rhinoceros (*Diceros bicornis*) that was moved from a tsetse-free to a tsetse-infested area in Kenya was monitored for trypanosome infection for two months following translocation. The animal acquired *Trypanosoma vivax* infection from a natural tsetse challenge, but survived without treatment. The infection was characterized by moderately high parasitaemia and symptoms of anaemia, leukopaenia and thrombocytopenia. Although confirmed to be *T. vivax* with deoxyribonucleic acid (DNA) hybridization tests, and development in the tsetse vector that was confined to only the proboscis, the trypanosomes had unusual morphology and motility. The isolate also failed to infect susceptible hosts such as cattle and goats.

## **1 Generation and validation of IgG isotype monoclonal antibodies for detection of *T. brucei*- and *T. congolense*-specific epitopes**

**Scientists:** R. Masake, T. Urakawa  
**Arch Associates:** S. Minja, C. Wells  
**Technicians:** J. Makau, A. Adema

Antigen-detection enzyme-linked immunosorbent assays (ELISA) developed for identifying animals infected with *Trypanosoma brucei* and *T. congolense* were based on monoclonal antibodies (MAbs) of IgM-isotype; namely, TR-7 and Tc-39, respectively. During field validation of these tests, the IgM MAbs appeared unstable, in contrast to the IgG<sub>1</sub> MAb Tv-27 (which detects an antigen of *T. vivax*). A semi-purified fraction of *T. brucei* antigens recognized by TR-7 was thus inoculated into BALB/c mice and IgG MAbs specific for *T. brucei* were generated (three IgG<sub>1</sub>, two IgG<sub>3</sub> and one IgG<sub>2a</sub>). One IgG<sub>1</sub> MAb, MAb T-43, was selected for use in antigen-detection ELISA. The MAb T-43 has a reaction pattern and sensitivity similar to MAb TR-7. By immunocytochemistry, MAb T-43 was shown to recognize an internal antigen located in the kinetoplast. However, MAb T-43 appeared not to react on Western blots.

In the case of *T. congolense*, protein bands of 38–40 kDa, reactive with the MAb Tc-39, were eluted from SDS-polyacrylamide gels and used to immunize BALB/c mice which were subsequently used in the generation of MAbs. Two IgG<sub>1</sub> and two IgM MAbs were obtained. IgG<sub>1</sub> MAb Tc-38 was selected on the basis of its sensitivity and reaction pattern. Monoclonal antibody Tc-38 reacts with antigens of 34–40 kDa of bloodstream trypomastigotes. Immunolocalization of the antigens revealed their presence in the kinetoplast and to a lesser extent in some lysosomes and chromatin. MAb Tc-38 was employed to detect *T. congolense* infection in experimentally infected cattle. MAb Tc-38 captured antigen in 38, 69, 76 and 80% of samples examined from four calves, compared to the currently used MAb Tc-39 which detected *T. congolense* antigen in 38, 71, 77 and 82% of the same samples. All the antigen-detection ELISA results obtained so far using the new IgG<sub>1</sub> MAbs against either trypanosome species give results comparable to the original IgM MAbs. Nevertheless, the MAbs will be subjected to a limited field validation prior to incorporation into Ag-ELISA kits.

### **T1b.2 Development of a PCR test for *T. vivax* based on *Duttonella*-specific DNA primers**

**Scientists:** R. Masake, P. Majiwa, S. Mooloo, O. ole-MoiYoi  
**Technician:** J. Njuguna

Recently, a cDNA, pcDSIL-800, encoding a *Trypanosoma vivax* diagnostic antigen was identified, cloned and sequenced. The gene is tandemly repeated, with a monomeric unit length of 900 bp, in the genomes of all *T. vivax* examined so far, isolated from diverse geographical areas in Africa and South America. In the absence of a DNA probe which hybridizes equally well to both West and East African isolates

of *T. vivax*, oligonucleotide primers based on the DNA sequences of pcDS have been designed and their potential use in the polymerase chain reaction for detecting *T. vivax* infection in livestock has been determined. Several primers were generated but only two (DP1 and DP2) proved useful. These primers amplified *T. vivax*-specific DNA sequences of *T. vivax* isolates from Uganda, Côte d'Ivoire, Nigeria, Colombia and French Guiana. The primers were used to amplify DNA from blood sequentially obtained from cattle exposed to tsetse-transmitted infection with *T. vivax* isolates from Kenya and Nigeria. The primers DP1 and DP2 amplified *T. vivax* DNA in samples collected five to seven days after tsetse challenge, as opposed to the first parasitological detection of trypanosomes which occurred on days 7 to 11 of infection. All the samples (except one) with detectable trypanosomes gave the expected PCR product, which hybridized with the pcDSIL-800 DNA probe. Blood samples from the experimentally infected animals remained positive by PCR until the parasites were no longer demonstrable by the buffy-coat phase-contrast technique for detection of parasites. Sera from the samples subjected to PCR were also examined for the presence of *T. vivax*-specific antigen by antigen-detection ELISA. Trypanosome-specific antigens appeared in the blood circulation either at the same time as the first PCR-positive reaction, or two to five days later. Out of the 162 samples examined, 70 (43%), 123 (76%) and 90 (56%) were positive by the buffy-coat technique, PCR and antigen-ELISA, respectively. Hence, the PCR was the most sensitive test.

### T1b.3 Characterization of trypanosome isolates from Burkina Faso

**Scientists:** R. Masake, P. Majiwa, G. Duvallet\*

**Technician:** A. Adema

\*CIRDES, Burkina Faso

Detection of trypanosome infections in tsetse flies can be achieved by dissection and microscopical examination of mouth-part contents, midgut contents or salivary glands. Alternatively, DNA probes recognizing DNA sequences unique to trypanosomes can be used for hybridization. The latter method (P. Majiwa and L. Otieno (1990), *Molecular Biochemical Parasitology* 40: 245-254) was employed by Duvallet and colleagues to characterize trypanosomes isolated from Burkina Faso. As reported before, seven of the trypanosome isolates (namely Samo-24-3, Sam 30/2, Sam/82/CRTA/33-4, C6, Sam 31/c1, KAA/83/CRTA/69 and Sam 29/2), hybridized with *Trypanosoma congolense* Savannah, Kilifi and Forest-type probes. Subsequently, the trypanosomes were cloned and the clones separately hybridized with the three DNA probes. Six out of seven clones derived from the isolates named above reacted with only the Savannah-type *T. congolense* DNA probe, while one (a derivative of C6) gave a positive signal with the Forest-type *T. congolense* DNA probe only. The DNA of the seven clones was separately amplified with an arbitrary primer known to give a distinct fingerprint pattern for Savannah, Kilifi and Forest-type *T. congolense*, respectively. The DNA fingerprint pattern of the clone derived from C6 was similar to that of *T. congolense* IL 3874, a Forest-type *T. congolense*, while the

rest of the clones displayed a pattern similar to that of Savannah-type *T. congolense*. Examination of the chromosome profiles of the seven clones revealed that each of them probably belongs to a different serodeme. The karyotype of the derivative of C6 was identical to that of IL 3874. These studies revealed that the isolates contained more than one type of *T. congolense*.

#### **T1b.4 Sensitive detection of *T. vivax* by DNA probe hybridization**

**Scientists:** P. Majiwa, R. Masake

**Technician:** J. Makau

Whilst PCR is becoming a valuable tool for the species-specific identification of trypanosomes, a simpler, direct hybridization of a trypanosome-specific DNA probe may be a more appropriate detection method at the present for use by some laboratories. The most suitable DNA probes are those capable of detecting small numbers of infective organisms. A cDNA of the transcript encoding the *T. vivax* species-specific diagnostic antigen has recently been identified, cloned and sequenced. The gene is present in the genomes of *T. vivax* isolates obtained from dispersed locations. The cDNA of the *T. vivax* diagnostic antigen has been used to screen a *T. vivax* genomic library, and thus identify a recombinant phage clone containing at least 15 kb of the genomic DNA within the vicinity of this gene. The genomic clone contains a tandem array of at least ten copies of the *T. vivax* diagnostic-antigen gene, units of which are 900 bp. Multimers of this gene have been transferred to a plasmid so that it can be evaluated in terms of its ability to detect, by hybridization, low numbers of *T. vivax* in the tsetse-fly mouth parts or the blood of an infected animal.

**T1c.1 Characterization of homidium uptake in *Trypanosoma congolense***

**Scientists:** A. Peregrine, J. Wilkes, D. Zilberstein\*

**Technicians:** J. Odhiambo, S. Kemei

\*Department of Biology, The Technion, Haifa, Israel

Homidium and isometamidium are both phenanthridine compounds that are widely used as trypanocides in cattle, sheep and goats. In earlier work the uptake kinetics of  $^{14}\text{C}$ -homidium were characterized in a homidium-sensitive clone of *Trypanosoma congolense*: IL 1180. Uptake was saturable and was inhibited, in part, by the purine nucleoside adenosine and guanosine. Over the past year the physiological characterization of the homidium-uptake process has been completed in *T. congolense* IL 1180. In contrast to adenosine and guanosine, neither the aminopurine adenine nor the nucleoside inosine inhibited uptake of  $^{14}\text{C}$ -homidium. However, nitrobenzylthioinosine (NBTI), an inhibitor of nucleoside transport, inhibited uptake of  $^{14}\text{C}$ -homidium by approximately 50% at a concentration of 100  $\mu\text{M}$ . The same concentration has previously been shown to inhibit uptake of  $^{14}\text{C}$ -Samorin in *T. congolense* IL 1180 by approximately the same amount. In order to determine the energy of activation of the homidium-uptake process, the uptake kinetics of  $^{14}\text{C}$ -homidium were determined at 2.5  $^{\circ}\text{C}$  intervals from 7.5  $^{\circ}\text{C}$  to 37.5  $^{\circ}\text{C}$ . The same was also determined for  $^{14}\text{C}$ -Samorin uptake, and demonstrated that the energy of activation for homidium was three-times greater than that for Samorin.

Finally, since homidium, isometamidium and the trypanocides diminazene and quinapyramine are chemically closely related, experiments were carried out to determine if the transport mechanism responsible for uptake of homidium is also responsible for uptake of other trypanocides. Thus, experiments were carried out to determine if isometamidium, diminazene or quinapyramine inhibit uptake of  $^{14}\text{C}$ -homidium in *T. congolense* IL 1180. At a concentration of 4.3  $\mu\text{g}/\text{ml}$ , neither diminazene nor quinapyramine had a significant inhibitory effect on uptake of  $^{14}\text{C}$ -homidium. However, isometamidium inhibited uptake by 79%. In a similar experiment, neither diminazene nor quinapyramine at a concentration of 4.3  $\mu\text{g}/\text{ml}$  inhibited uptake of  $^{14}\text{C}$ -Samorin. However, homidium inhibited uptake by 72%. Collectively, therefore, these data indicate that the transporter mechanism responsible for uptake of homidium in *T. congolense* IL 1180 is also responsible for uptake of isometamidium.

**T1c.2 Cross-resistance phenotype associated with induction of resistance to isometamidium chloride (Samorin<sup>®</sup>) in *Trypanosoma congolense***

**Scientist:** A. Peregrine

**Technician:** S. Kemei

Isometamidium chloride (Samorin<sup>®</sup>), diminazene aceturate (Berenil<sup>®</sup>), homidium chloride (Novidium<sup>®</sup>) and quinapyramine sulphate (Trypacide sulphate<sup>®</sup>) are all

anti-trypanosomal compounds that are routinely used in domestic livestock; quinapyramine sulphate is restricted to use in *equidae*. Because the four compounds are closely related chemically, cross-resistance has been suggested to occur between a number of them. However, a clear definition of the cross-resistance relationship has not been possible due to the lack of trypanosome populations in which resistance to individual trypanocides has been unequivocally induced. *Trypanosoma congolense* IL 1180 is a cloned population that expresses a high level of sensitivity to isometamidium chloride *in vivo* and *in vitro*; in mice the dose required to cure 50% of infected animals (CD<sub>50</sub> value) is 0.018 (95% confidence interval = 0.013–0.025) mg/kg bodyweight (b.w.). In earlier work, repeated sub-curative treatments of infected mice with isometamidium chloride was used to increase the isometamidium chloride CD<sub>50</sub> value of IL 1180 to 1.7 (1.4–2.0) mg/kg b.w. In order to determine whether induction of resistance to isometamidium results in cross-resistance to other trypanocides, the parental clone and isometamidium-resistant derivative were characterized in mice for their sensitivity to diminazene aceturate, homidium chloride and quinapyramine sulphate. With the parental clone the diminazene aceturate, homidium chloride and quinapyramine sulphate CD<sub>50</sub> values were 2.3 (2.0–2.6) mg/kg b.w., 0.37 (0.3–0.4) mg/kg b.w. and 0.23 (0.15–0.34) mg/kg b.w., respectively. In contrast, the values for the isometamidium-resistant derivative of IL 1180 were 7.8 (7.5–8.1) mg/kg b.w., 12.1 (5.8–25) mg/kg b.w. and 0.97 (0.84–1.1) mg/kg b.w., respectively. Thus, an approximately 94-fold increase in resistance to isometamidium was associated with a high level of cross-resistance to homidium and low levels of cross-resistance to diminazene and quinapyramine. In a similar manner to studies on the uptake of isometamidium and homidium by trypanosomes, these data suggest that resistance to isometamidium and homidium in *T. congolense* is mediated, at least in part, by the same mechanism.

### **T1c.3 The pharmacology of Samorin: use of separation technology**

**Scientist:** J. Wilkes

**Research Fellow:** C. Patel\*

\*Department of Pharmacology, University of Glasgow, UK

Samorin, one of the major drugs utilized in the treatment of animal trypanosomiasis in sub-Saharan Africa, is a complex mixture of closely related compounds, demonstrating a range of trypanocidal activities. The mixture may be qualitatively demonstrated by thin-layer chromatography. Although the existence of the major compounds in the preparation (a red form [isometamidium chloride] and a purple form of lower trypanocidal activity) were described in the early sixties, there is very little information available on the pharmacology and biotransformations of the drug.

An isocratic, reverse phase high performance liquid chromatography (HPLC) system capable of separating the components of Samorin has been devised. The only previous HPLC system for native Samorin was described by Kinabo and

Bogan (*Acta Tropica* 45: 165–170, 1988). This utilized an ion-pairing system of sodium heptane sulphonic acid and tri-ethylamine (TEA) in methanol-water mixtures. The methodology detected Samorin by fluorescence in the column eluent, and reported only a single peak. We have modified the original method to effect the separation and detection of the components. Absorption is monitored at 380 nm, as only two components of Samorin show significant fluorescence; these being two positional isomers of isometamidium chloride. An extensively deactivated column packing, changed from a standard ODS (C-18) column, gives much improved resolution of strongly basic compounds. Finally, the capacity factors for the various components, and hence the ability to separate them, were found to be highly sensitive to the water content of the solvent system.

The optimized HPLC system used utilized Na-heptane sulphonic acid (20 mM), TEA-H<sub>3</sub>PO<sub>4</sub> (0.14% w/v, pH 4) and 67% methanol. This system effectively separated the components of Samorin, except for the two positional isomers of isometamidium chloride which co-eluted. A series of HPLC systems were designed and tested in order to attempt the separation of these components. All systems were variations on the ion-pairing methodology, utilizing changes in the nature of the ion-pairing compound, the concentrations of the various components, and the flow rates. One major benefit was the substitution of pentane-sulphonic acid for heptane sulphonic acid, which improved the capacity factors and halved the time for complete elution of Samorin components to five to six minutes. No effective separation of the positional isomers was demonstrated.

In complementary studies, a proprietary gradient HPLC system provided by Rhône Mérieux was utilized to isolate <sup>14</sup>C-labelled components of Samorin. These components were then used to study the uptake of the various compounds by *Trypanosoma congolense* IL 1180. Initial studies indicated that the positional isomers and the purple form are all transported into bloodstream form IL 1180, but that two separate transport systems are present.

Finally, a bulk method for the enrichment of Samorin's components was developed, resulting in yields of 10s to 100s of milligrams, based on a counter-current distribution of the drug in butan-1-ol-water phase separations in the presence of saturating concentrations of Na<sub>2</sub>SO<sub>4</sub>. This method exploits the different partition coefficients of the components in these phase systems (ranging from 10 to 0.03), so improving the separation by repeated transfer of the upper phases to fresh lower phase (analogous to the mobile and stationary phases in conventional chromatography).

The reverse phase-HPLC separation system will be used to study the pharmacokinetics of the various Samorin components, and to investigate the adducts, breakdown products and metabolites of the drug in both host and parasite. The availability of enriched fractions of the components of Samorin will allow a precise characterization of the uptake mechanism in trypanosomes.

**T1c.4 Preparation of membrane vesicles of *Trypanosoma brucei brucei* and their transport activities****Scientists:** J. Wilkes, D. Zilberstein\***Research Associate:** C. Wells**Technicians:** J. Odhiambo, S. Kemei

\*Department of Biology, The Technion, Haifa, Israel

*Trypanosoma brucei brucei* (ILTat 1.4) bloodstream forms isolated from infected rats were subjected to various methods of cell rupture including nitrogen cavitation, French press, sonication and glass bead abrasion. Of the four methods studied, only glass bead abrasion appeared to produce sealed membrane vesicles. The membrane vesicles were isolated by differential centrifugation and pelleted from the supernatant at 105,000 g for one hour. The resuspended pellet was examined by electron microscopy and showed a high proportion of homogeneous closed membrane vesicles; the variable surface glycoprotein could be visualized on the surface of the vesicles, suggesting that the vesicles were derived from the plasma membrane of trypanosomes and were right side out.

The transport activity of the membrane vesicles was monitored by comparing the uptake of radiolabelled glucose (<sup>3</sup>H-glucose) with the transport of glucose across rabbit intestinal brush-border membrane vesicles; the uptake was linear for the first minute. There was also an increase in uptake with an increase of temperature from 4 °C to 37 °C. Finally, the concentration of Na<sup>+</sup> and K<sup>+</sup> ions in the medium had no effect on the uptake of glucose, in contrast to rabbit intestinal brush-border membrane vesicles. This implies that uptake of glucose by *T. b. brucei* membrane vesicles is not an ion-linked process, but is a membrane-mediated transport process. These vesicles will be used to study the transport of drugs across the plasma membrane of trypanosomes.

**T1c.5 Long-term persistence of multiple-drug resistant populations of *Trypanosoma congolense* in cattle at Ghibe, Ethiopia****Research Fellow:** W. Mulugeta**Supervisors:** A. Peregrine, J. Wilkes, R. Masake

In July 1989 12 trypanosome isolates were collected from cattle at Ghibe, Ethiopia. When characterized for their drug sensitivity in cattle, all 12 populations were resistant to diminazene aceturate at a dose of 7.0 mg/kg bodyweight (b.w.). Eleven of the 12 populations were also resistant to isometamidium chloride and homidium chloride at doses of 0.5 and 1.0 mg/kg b.w., respectively. This suggested that there was a high prevalence of multiple-drug resistant infections at Ghibe, and that chemotherapy *per se* would not control trypanosomiasis. A tsetse-control programme was therefore initiated in April 1990 (See Abstract T1a.3). Over the subsequent 12-month period this was associated with a significant reduction in both the apparent

density of tsetse flies and the prevalence of trypanosome infections in cattle. A decrease in the rate of relapse of infections in cattle after treatment with diminazene aceturate was also observed. In order to determine whether these observations were associated with an alteration in the drug-resistance phenotype of trypanosome populations, a second set of trypanosome isolates was collected from cattle at Ghibe in February 1993; ten isolates were collected at random. All 10 populations were Savannah-type *Trypanosoma congolense*. When their drug sensitivity was assessed during infections of Boran (*Bos indicus*) calves, all 10 isolates were found resistant to diminazene aceturate, isometamidium chloride and homidium chloride when administered intramuscularly at doses of 7.0 mg/kg b.w., 0.5 mg/kg b.w. and 1.0 mg/kg b.w., respectively. In order to determine whether this multiple-drug resistance was expressed by individual trypanosomes, clones were derived from two of the isolates which grew in mice. The clones were subsequently characterized for their sensitivity to the aforementioned trypanocides in mice. Clones IL 4009 1 and IL 4015.1 had diminazene aceturate 50% curative dose ( $CD_{50}$ ) values of  $45.9 \pm 0.13$  (SD) mg/kg b.w. and  $30.0 \pm 0.25$  mg/kg b.w., respectively. Both clones had homidium chloride  $CD_{50}$  values that were in excess of 20 mg/kg b.w. Finally, the isometamidium chloride  $CD_{50}$  values for the two clones were  $12.5 \pm 0.21$  mg/kg b.w. and  $9.8 \pm 0.16$  mg/kg b.w., respectively. These data therefore indicate that both clones express high levels of resistance to all three trypanocides. In experiments carried out to characterize the uptake kinetics of  $^{14}C$ -Samorin, the maximal rates of uptake ( $V_{max}$ ) for four Ghibe isolates collected in 1993 ranged from 9.2 to 15.0 ng/ $10^8$  trypanosomes/minute. In contrast,  $V_{max}$  for the drug-sensitive clone *T. congolense* IL 1180 was  $86.7 \pm 8.6$  ng/ $10^8$  trypanosomes/minute. For three of the Ghibe isolates and IL 1180, the uptake data were fitted by an allosteric model. However, only the three Ghibe isolates demonstrated high level cooperation. Data for the fourth Ghibe isolate was best modelled using Michaelis-Menten kinetics. Lastly, molecular karyotype profiles were determined for eight isolates and demonstrated eight different profiles. These data therefore indicate that in February 1993 there was a number of genetically distinct populations at Ghibe that expressed high levels of multiple-drug resistance. Since a similar situation existed at the same site in July 1989 populations of drug resistant trypanosomes continue to be maintained in Ghibe.

### T1c.6 Application of an ELISA for isometamidium

**Research Fellow:** M. Eisler\*

**Scientists:** A. Peregrine, P. Holmes\*, S. Mooloo, J. Maruta†, R. Connor‡

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Two robust ELISA procedures, one an indirect-competition ELISA, the other a competitive enzyme immunoassay, have been developed for the detection and quantification of isometamidium in the serum of treated cattle. The ELISAs have been used at ILRAD to investigate the relationship between concentrations of isometamidium circulating in Boran cattle, and prophylaxis against tsetse challenge with stocks of *T. congolense* (IL 3893, IL 3889, IL 3343 and IL 1180) of various sensitivities to the drug. Following intramuscular (i.m.) injection of isometamidium chloride (Samorin®) at a dose rate of 1.0 mg/kg<sup>-1</sup> bodyweight, cattle were repeatedly challenged by the application of *T. congolense*-infected *Glossina morsitans centralis* at monthly intervals, until breakthrough infections occurred. The infectivity of challenge was confirmed using untreated control cattle. All ten cattle challenged with *T. congolense* IL 3893 (five cattle) or IL 3889 (five cattle) developed infection following the first challenge, at which time the mean drug concentration was 6 ng/ml<sup>-1</sup>. At the first challenge, one month after drug administration, all ten cattle challenged with IL 3343 were refractory to infection. However, all succumbed to challenge at the second (seven cattle) or third (three cattle) monthly challenges. The mean isometamidium concentration at the time of the second challenge was 2 ng/ml<sup>-1</sup>, and concentrations were not significantly different between cattle refractory to infection with IL 3343 and those that succumbed to infection. Cattle challenged with IL 1180 became infected following six to eight monthly challenges, by which time isometamidium concentrations could no longer be detected. In uninfected cattle at the time of the third and fourth monthly challenges, the drug concentration means were 0.75 and 0.4 ng/ml<sup>-1</sup>, respectively. Trypanosome populations IL 3893 and IL 3889 were considered to be highly resistant to isometamidium, IL 3343 to be of moderate resistance and IL 1180 to be relatively sensitive. It was therefore concluded that when *T. congolense* are found to persist in the circulation of cattle in which detectable levels of isometamidium are present, the following inferences can be made: at isometamidium concentrations greater than 6 ng/ml<sup>-1</sup>, *T. congolense* can be considered markedly resistant; between 2 and 6 ng/ml<sup>-1</sup>, resistance may be considered to be at least moderate; between 0.4 and 2 ng/ml<sup>-1</sup>, only a low level of resistance may be inferred; below 0.4 ng/ml<sup>-1</sup> no inferences regarding drug resistance may be made. These results will enable the isometamidium ELISA to be used to investigate isometamidium resistance of *T. congolense* in the field.

An experiment was conducted in Zimbabwe in collaboration with the Regional Tsetse and Trypanosomiasis Control Programme (RTTCP) and the Department of

Veterinary Services. It successfully demonstrated that the measurements previously obtained in the laboratory could be reproduced in cattle kept under field conditions and exposed to natural tsetse challenge. Twenty-four cattle from the low tsetse-challenge area of Makuti on the Zambezi escarpment received diminazene aceturate (7.0 mg/kg<sup>-1</sup> bodyweight i.m.) and were moved two weeks later (15 February 1993) to Rekomitjie, a tsetse-infested area in the Zambezi valley. Isometamidium chloride was administered i.m. at a dose rate of 1.0 mg/kg<sup>-1</sup> bodyweight on arrival. The cattle were then maintained at Rekomitjie for six months. Packed red cell volume, parasitological status (buffy-coat and blood smear techniques) and isometamidium concentrations (ELISA) were determined fortnightly. Isometamidium treatment effectively prevented trypanosome infections in the majority of cattle over most of the six-month period of the experiment; only three cases of trypanosome infections were detected in treated cattle, none until 140 days after isometamidium administration. In contrast, in a group of 18 untreated cattle in the same area, nine cases occurred over the same period: none in February, two in March, one in April, four in May, two in June and none in July. Serum isometamidium concentrations, the rate of drug elimination and protection against *T. congolense* challenge were similar to results of cattle experiments in Glasgow and ILRAD when cattle were challenged with isometamidium-sensitive populations, i.e., four to five months protection after a single treatment with 1.0 mg isometamidium chloride/kg<sup>-1</sup> bodyweight. Isometamidium concentrations as low as 0.4 ng/ml<sup>-1</sup> appeared to be sufficient to protect the majority of cattle against trypanosome challenge. There was therefore no evidence of isometamidium resistance at the study site.

### **T1c.7 Induction and characterization of a quinapyramine-resistant clone of *Trypanosoma congolense***

**Research Fellow:** G. Ndoutamia

**Scientists:** N. Murphy, A. Peregrine

A quinapyramine-resistant clone of *Trypanosoma congolense*, IL 118G, has been derived that is approximately 40-times more resistant to quinapyramine than the parental clone. This increase in resistance was shown to be associated with significant cross-resistance to isometamidium, homidium and diminazene in mice and goats. Using random primers in a polymerase chain reaction (PCR), differences have been identified between the parental clone and the 40-fold resistant population. Furthermore, two primers (ILo 868 and ILo 1122) revealed differences that appeared to correlate with the increasing levels of resistance when populations with intermediate levels of resistance were included in the analyses. While the PCR using ILo 868 was not consistently reproducible, three of the PCR products (0.4 kb, 0.8 kb and 1 kb) generated with ILo 1122 were shown to consistently vary between the sensitive and resistant populations. For further analysis the three PCR products were cloned into the plasmid pBluescript. Hybridization to Southern blots of separated chromosomes revealed that the 1 kb and the 0.4 kb product of primer ILo 1122 hybridize to three different chromosomes, while the 800 bp product

hybridizes only to one chromosome to which the two other products also hybridize. The 800 bp product of ILo 1122 has been completely sequenced. The actual size of this product is 782 bp and the entire sequence consists of 30 bp tandem repeats. No differences were observed between the sensitive and resistant populations by Southern blot analysis of digested genomic DNA hybridized with the 800 bp product of ILo 1122. It is believed that the consistent differences observed in the PCR may be due to point mutations at the priming site in the genomes of the different trypanosomes.

Northern blot analysis indicated that the 800 bp product of ILo 1122 hybridizes to a transcript of 2.3 kb which is expressed at similar levels in the drug-resistant and sensitive populations. The 800 bp product was therefore used to screen a *T. congolense* cDNA library. Three clones of 3.0 kb were isolated. All the clones cross-hybridized to each other and had two internal *Eco* RI sites. The three fragments (1.6 kb, 1 kb, 0.4 kb) generated by *Eco* RI digestion were subcloned into plasmid pBluescript.

In another experiment, the 800 bp product of ILo 1122 was hybridized to a Southern blot of an *Eco* RI digest of the 3 kb cDNA. The results showed that the 800 bp product hybridizes only to the 1.6 kb *Eco* RI products of the cDNA. To date, 1398 bp of the 1.6 kb fragment have been sequenced.

Experiments are under way to amplify the ends of the 800 bp sequence by PCR from DNA isolated from sensitive and resistant populations and sequence this region to elucidate the nature of the suspected mutations.

### **T1c.8 Genomic libraries of DNA from *Trypanosoma vivax*, *T. simiae* and different genotypic groups of *T. congolense***

**Scientist:** P. Majiwa

**Technician:** M. Maina

Genomic libraries have been made from DNA of *Trypanosoma vivax* ILDat1.2, *T. congolense* IL 1180, *T. congolense* K45.1, *T. congolense* IL 3900, *T. congolense* CP1091 and *T. simiae* KETRI 2431/1. DNA from each trypanosome was partially digested with a restriction enzyme (*Sau*3A1) and separated into size-classes by sedimentation in gradients of sucrose. The gradients were fractionated and the size of the DNA fragments in each of fractions determined. For the construction of genomic libraries, DNA fragments of 10 to 20 kb were recovered and ligated into *Bam*H1 arms of  $\lambda$ GEM-11 which were subsequently packaged into phage particles. To test the potential utility of these libraries and to determine the size distribution of the DNA fragments inserted in the recombinant phages, each of the libraries was screened for the gene encoding ribosomal RNA. The rDNA genomic clones identified in each library have inserts ranging in size from 8 to 18 kb. Finally, the genomic library of *T. vivax* was screened for the genomic clone containing the gene which encodes the *T. vivax* diagnostic antigen. These libraries will serve as useful resources for genetic mapping, transfection experiments and inter-species homology searches for potential resistance genes in the future.

### **T1c.9 Ribosomal RNA genes of the *Nannomonas* trypanosomes**

**Scientists:** T. Urakawa, P. Majiwa

**Technician:** M. Maina

One of the impediments to effective genetic studies of African trypanosomes is the lack of a system for efficient and routine transformation of these parasites. Although *T. brucei* can be transformed in a fairly straight forward manner, this is not the case with *T. congolense* and *T. vivax*. As part of an effort to identify loci within the *T. congolense* genome into which exogenous genes can be inserted and thus be brought under the control of endogenous strong promoter(s) *in cis*, comparative analyses are being performed of the loci containing both the large and the small subunit (LSU and SSU) ribosomal RNA genes of the *Nannomonas* trypanosomes. Genomic clones have been identified which contain one complete SSU and a segment of the LSU of *T. congolense* IL 1180, *T. congolense* K45.1, *T. congolense* IL 3900, *T. congolense* CP1091, *T. simiae* KETRI 2431/1 and *T. vivax* ILDat 1.2.

The SSU gene in each of the genomic clones has been transferred to plasmids for sequence determination. So far, the nucleotide sequence has been determined for the entire SSU of a Savannah-type *T. congolense* and a Kilifi-type *T. congolense*. The Kilifi-type *T. congolense* SSU is 93% similar in nucleotide sequence to that of the Savannah-type *T. congolense*, and the Kilifi-type *T. congolense* or the Savannah-type *T. congolense* SSU are each only 86.5% similar in sequence to that of *T. brucei*. Work is in progress to determine the nucleotide sequence composition of the complete SSU of Tsavo-type *T. congolense*, the West African riverine/forest-type *T. congolense* and *T. simiae*. Information so obtained will facilitate (a) the identification of sequences which can be used to build plasmid constructs for general transformation of the different genotypic groups of *T. congolense*, (b) determination of the location within the rDNA into which exogenous DNA can be inserted, (c) design of oligonucleotide primers for the specific reverse transcriptase PCR amplification of rRNA of different species of trypanosomes, and (d) the establishment of the phylogenetic relationships of the different types of *T. congolense*.

### **T1c.10 *Trypanosoma simiae*: studies of drug susceptibility *in vitro***

**Scientists:** E. Zwegarth\*, S. Moloo, R. Kaminsky†

**Technicians:** J. Mulati\*, C. Sabwa, J. Kabata, J. Muia

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Two stocks of *Trypanosoma simiae* were initiated in culture with tsetse-derived metacyclics. Thereafter, they were propagated axenically as trypomastigote forms at 35 °C in 4% CO<sub>2</sub> in air. Populations of trypomastigotes were then incubated with various concentrations of anti-trypanosomal compounds; growth was monitored after 24 hr of incubation by counting trypanosome numbers, and the growth inhibition

calculated by comparing values with the same trypanosome populations that were incubated for the same period in the absence of the compounds. Diminazene aceturate, quinapyramine sulphate, DL- $\alpha$ -difluoromethylornithine and Ro 15-0216 (a nitroimidazole) showed activity against the two stocks of *T. simiae*. Suramin and melarsomine had little effect upon the growth of the parasite populations. Finally, isometamidium chloride gave questionable results in the 24 hr growth inhibition test, but the results of a ten-day, long-term viability assay indicated some degree of drug resistance. The results correlate with the observations obtained with the same populations when their drug sensitivity was characterized in pigs. Thus, it is concluded that in many cases the cryptic nature of *T. simiae*, rather than drug resistance, is responsible for the failure of chemotherapy of trypanosomiasis due to *T. simiae* in pigs.

**T2a.1 Application of the RADES-PCR method for the identification of many differentially expressed sequences in metacyclic and bloodstream forms of *Trypanosoma congolense* IL 3000****Scientists:** N. Murphy, R. Pellé**Technicians:** P. Pandit, A. Muthiani

The availability of axenic culture systems for the propagation and isolation of pure populations of all life-cycle stages of *Trypanosoma congolense* and the recent development of a differential display method at ILRAD, called RADES-PCR, for the rapid identification of developmentally expressed genes, now allows for the detailed analysis of molecular events associated with the switch from non-dividing infective metacyclic insect forms to actively-dividing bloodstream forms of *T. congolense*. Understanding the molecular events involved in this switching process and the availability of molecular markers to follow it both *in vitro* and *in vivo* will allow for the dissection of early processes of importance in the onset of an infection. Observations from infections in trypanotolerant and susceptible cattle suggest that there are likely to be important differences in responses very early during an infection, possibly during the differentiation from metacyclic to bloodstream forms.

Using the RADES-PCR differential display method, approximately 250 PCR products derived from differentially expressed genes in metacyclic and established bloodstream form parasites have been isolated. These products are currently being cloned, characterized and mapped and additional studies are under way to examine differential gene expression during the transition from metacyclic forms to early, and then late, bloodstream forms. To date, over 30 of the products have been cloned and sequence information derived from 20. Detailed analysis of the sequenced products is currently under way to determine whether they encode sequences which have homologues in the current sequence databases. First pass sequencing to date has resulted in the identification of a *c-fos* oncogene homologue, a cyclin B homologue (which in other systems is central to the onset of mitosis and cellular proliferation), a ribosomal protein homologue and potential receptor molecules, all of which are specific to the actively-proliferating bloodstream form. However, the majority (> 80%) of characterized sequences do not identify homologues in the DISA sequence databases. Cross-hybridization analysis between *T. brucei brucei* and *T. congolense* suggests that there are copies of many of these sequences in both parasite species. Some of the cloned sequences have been supplied to Dr. S. Melville of Cambridge University, UK, to test against a P1 library and chromosome blots of *T. brucei* to assist in the genetic mapping of this trypanosome species.

## **T2a.2 Further improvements in the RADES-PCR method for the easier and more rapid characterization of developmentally expressed genes**

**Scientists:** N. Murphy, R. Pellé

**Technician:** P. Pandit

The recently developed differential display method called RADES-PCR (for randomly amplified developmentally expressed sequences-PCR) is proving to be an exceptionally sensitive and powerful method for molecular studies on trypanosome differentiation. A limiting step in the technique is the time consuming processes of cloning each differentially amplified product, screening of *Escherichia coli* bacteria for the cloned products, growth and subsequent purification of plasmids from potential clones of interest, followed by the derivation of the DNA sequence of the cloned insert. Each of the above steps has therefore been examined to determine where improvements could be made to more effectively exploit the potential of the RADES-PCR method. Strategies for cloning PCR products in many instances result in relatively large numbers of white colonies which do not contain recombinant plasmids (false positives). Hence, between five and ten colonies from each cloning experiment require testing before a true positive clone is identified. Oligonucleotide primers for sequencing through the multiple cloning sites in these plasmid vectors can be used to directly screen white bacterial colonies to determine whether the endogenous plasmids have cloned inserts of the correct size. The availability of 96-well PCR thermocyclers allows for the screening of 95 white colonies and one control in a single experiment. PCR products are analysed on agarose gels and reactions generating products of the correct size are chosen for direct sequence analysis. Only 1 µl of the products from each of the screening PCR reactions is required to carry out a sequencing reaction by the *fmoI*<sup>TM</sup> PCR sequencing procedure (Promega). Sequence information from both strands of the cloned DNA fragment can be derived from two sets of sequencing reactions. The DNA sequence information produced is as clean as that produced from purified plasmid DNA and yet requires a fraction of the time to generate.

Direct sequence analysis of the differentially amplified PCR products, without the cloning step, would be a further substantial improvement in the technique. However, since only one short oligonucleotide primer (a 10-mer) is used to generate each RADES-PCR fingerprint, sequencing of one DNA strand is not possible. Further, short oligonucleotide primers have insufficient specificity to generate clear and reliable DNA sequence information. An alternative priming strategy is required to generate a product which differs in sequence at either end and which can be directly sequenced. To date, all trypanosome mRNA transcripts analysed display a fixed 5' spliced-leader sequence of 39 nt. A set of four oligonucleotides were generated which consisted of the most 3' 11 nucleotides of the spliced-leader sequence, a mixed nucleotide (A, C, G and T) and either an A, G, C or T nucleotide at the 3' end. The function of the last two nucleotides was firstly to anchor each primer to give a fixed end and secondly to select for only a portion of the cDNA molecules to reduce the complexity of the fingerprint patterns. This would also effectively increase the usefulness of each arbitrary primer four-fold, since a combination of four different

sets of primers could be utilized. In test experiments this was found to be the case, indicating that the majority of products were generated by an arbitrary primer at one end and one of the four anchor primers for each reaction at the other end. Using the anchor primers alone, only a small amount of sequence information, which was difficult to clearly read, was generated. Although the anchor primers are longer than the arbitrary primers, they are still too short for the specificity of priming required to generate reliable sequence information. An alternative strategy has been tested in which a longer primer against the spliced-leader sequence is used for generating the sequence information. A universal spliced leader sequence primer (a 34-mer) was synthesized and used in the *fmol*<sup>TM</sup> PCR sequencing reaction with the first five rounds of PCR carried out at a low annealing temperature and the subsequent 30 rounds at a higher, more restrictive, annealing temperature. Not all PCR products generated with an anchored spliced-leader and arbitrary primer are appropriate templates for direct sequence analysis. However, those that are can be identified by first carrying out a test PCR reaction to determine whether a specific product is generated and then utilize ones that give a positive result for further sequence analysis. Products which demonstrate a lower fidelity of amplification can be cloned and characterized as outlined above. An additional advantage of this approach is that the DNA strand containing an open reading frame is readily identifiable. The combination of these modifications substantially improves the rate at which differentially amplified products can be characterized.

**T2a.3 The RADES-PCR method identifies differences in gene expression between procyclic forms of *Trypanosoma congolense* IL 1180 derived *in vitro* or *in vivo***

**Research Fellow:** N. Chepkorir

**Scientists:** N. Murphy R. Pellé, S. Moloo

**Technicians:** P. Pandit, J. Wando, A. Muthiani

Expression of developmentally expressed genes in trypanosomes is significantly influenced by alterations in the external environment, such as temperature and nutrients. The different developmental stages of *Trypanosoma congolense* are propagated *in vitro* under different conditions and current systems can generate sufficient numbers for molecular and biochemical studies. However, conditions *in vitro* for the propagation of the different life cycle stages differ substantially from conditions *in vivo* and it is important to determine whether differentially expressed sequences identified from material generated *in vitro* also show the same expression profiles *in vivo*. Different life cycle stages of *T. congolense* IL 1180 from infected tsetse flies have been compared with similar stages generated *in vitro*. Initially the focus has been on procyclic forms (PCF), since these are the most abundant and easily purified life cycle stage in the tsetse fly.

Infected tsetse flies were dissected and parasites isolated by placing the infected midguts in tubes containing procyclic culture media followed by vortexing to release PCF parasites attached to the fly gut material. Low speed centrifugation was carried

out to remove dense tsetse fly material, followed by a higher speed centrifugation of supernatants to isolate the PCF parasites. The resultant pellets were then snap frozen in liquid nitrogen. The PCF parasites propagated *in vitro* were similarly pelleted and snap frozen. Total RNA was purified from both populations of parasites and analysed on an agarose gel. It was clear from the predominant ribosomal bands that the majority of the RNA isolated from the tsetse fly PCF parasites was in fact of host (tsetse fly) origin. Enrichment of poly(A)<sup>+</sup> RNA from both populations was carried out followed by first strand cDNA synthesis in the presence of reverse transcriptase and oligo(dT). A portion of both cDNAs was used as the template in PCR reactions designed to specifically amplify cDNA of trypanosome origin with primers which exploit the conserved 5' spliced-leader and 3' poly(A) sequences found on all trypanosome mRNAs. Following removal of these primers and dNTPs, the resultant double stranded cDNAs were used as templates for RADES-PCR analysis with 10-mer arbitrary primers. The majority of primers generated fingerprints which were similar or identical for both cDNA templates. cDNA from a second parasite clone, IL 3000, was also included in some of the RADES-PCR experiments. In addition to differences in some RADES-PCR products generated from material derived *in vitro* and *in vivo*, differences were also observed between products generated from the two clones, IL 1180 and IL 3000, propagated *in vitro*. To ensure that the RADES-PCR products were of trypanosome, rather than tsetse fly, origin, Southern blots of the products were probed with total labelled trypanosome genomic DNA. Further confirmation of the specificity of the RADES-PCR products specifically amplified from trypanosomes raised *in vitro* or *in vivo* was achieved through probing of Southern blots with labelled cDNA from the two sources. Some of the differentially amplified products were cloned and sequenced.

One of the specific products from trypanosomes from tsetse was of particular interest, since it displayed a significantly higher level of expression in the parasites generated *in vivo*, but did not display differential expression between the different life cycle stages generated *in vitro*, or between bloodstream forms and the other life cycle stages derived *in vivo*. Sequence analysis of the cloned product revealed an apparently bi-functional gene product with significant homology in one domain to the eukaryotic L19 ribosomal gene and in another domain to proteases, particularly serine proteases. A product preferentially expressed in cultured forms has also been sequenced, but no homologue has been found in the current DNA sequence databases.

#### **T2a.4 Development of a quantitative PCR method for the accurate and rapid estimation of parasite numbers**

**Senior Research Fellow:** J. Enyaru

**Scientist:** N. Murphy

**Technician:** D. Ndegwa

It is important to develop a simple, fast and more accurate method to quantify trypanosome numbers in infections, particularly early infections in cattle. This may facilitate, amongst other things, a more accurate definition of trypanotolerance and

the stage at which parasitaemia is controlled. The objective of the current study is to develop a competitive PCR-based method and test its accuracy in quantifying parasite numbers in early infections of N'Dama and Boran cattle. The method will utilize the diagnostic repetitive sequence of the Savannah group of parasites. A Plasmid, pUC9, containing a cloned fragment of this diagnostic sequence, p372M, was provided by Dr. P. Majiwa. Approximately four copies of the 369 bp diagnostic repeat sequence are contained within the cloned insert, which is a *T. congolense* *Sau3A* I fragment ligated in the *BamH* I site of pUC9. Since there is a *BstB* I restriction enzyme site within the repeat, and not in the plasmid vector, digestion of p372M should result in removal of all copies of the repeat, leaving both parts of one attached to the vector. Re-circularization of the cut plasmid should then generate a plasmid containing just one copy of the cloned repeat. Attempts to generate a plasmid containing just one copy of the repeat sequence by this procedure failed, since some copies lacked the expected *BstB* I site. A single 369 bp copy of the repeat has therefore been cloned through a complete *Sau3A* I digestion of *T. congolense* genomic DNA and ligation into the plasmid vector pBluescript. The sequence of the cloned insert is being verified. The cloned fragment will then be modified by deletion of internal sequences. The quantitative PCR technique will be based on seeding blood samples, containing parasites, with known quantities of the modified repetitive sequence. The technique will be initially developed to quantify parasite numbers from infections in rats prior to testing in N'Dama and Boran cattle. Standardization of the PCR method is under way using diagnostic primers for the amplification of the *T. congolense* repeat sequence.

**T2a.5 Studies on the timing of commitment of the metacyclic to bloodstream form switch and development of a system for the generation of *Trypanosoma congolense* parasites with altered virulence characteristics**

**Scientist:** N. Murphy

**Technician:** A. Muthiani

Metacyclic trypanosomes are non-dividing coated forms, which are considered to be pre-adapted for survival in the mammalian host. Metacyclic forms are often compared to non-dividing short stumpy (SS) bloodstream forms (BSF) of *T. b. brucei* which are also non-dividing and have been suggested to be pre-adapted for growth in the tsetse fly insect vector. However, since *T. b. brucei* SS forms display characteristics of a dying form, and do not survive unless switched to insect-like conditions, there is some debate as to whether this form is a true life cycle stage or a degenerate dying form. We have therefore examined whether metacyclic forms of *T. congolense* display similar characteristics to *T. b. brucei* SS forms.

Mature metacyclic forms of *T. congolense* IL 3000 were isolated by DE53 column chromatography and placed in either metacyclic or BSF media. Cultures were maintained at 27 °C and individual flasks switched to 37 °C on days 1 to 14. Parasites (1 to 2 × 10<sup>5</sup>) were also inoculated into irradiated mice on each day and the resultant parasitaemia monitored. Establishment of BSF cultures was monitored and growth

rates determined. Mice infected with these metacyclic forms generally survived for 10 to 14 days. It was noted that the principal trigger *in vitro* for the switch from metacyclic to BSF is temperature, and although parasites in metacyclic form media did not reach as high a cell density as those in BSF media, they did undergo active division and attachment. In general, results from the experiments *in vitro* concurred with those *in vivo*. Mice infected with metacyclic forms maintained at 27 °C for five to six days started to display non-lethal fluctuating parasitaemias, and, in some cases, these mice became aparasitaemic. Many dying forms of the trypanosomes were also observed in the cultures *in vitro* maintained at 27 °C for this period and then switched to 37 °C. Although many of the parasites maintained for longer periods at 27 °C could still produce lethal infections in mice, repeat experiments suggest that days 5 to 6 are a 'water-shed' for the survival of mature metacyclic forms. It was also found that this time point can shift for older metacyclic producing cultures. Three independent isolates of IL 3000 parasites causing fluctuating, non-lethal infections in mice have been recovered and will be re-tested in mice following their establishment in axenic culture systems.

Results suggest that the majority of mature metacyclic forms are, like in *T. b. brucei* SS forms, 'terminally differentiated' and die unless switched to appropriate conditions for the next life cycle stage. Further, those parasites that survive would appear to lack the appropriate signals for a type of 'programmed cell death' and the loss of this characteristic seems to be associated with a decreased virulence phenotype. The isolates which display a decreased virulence phenotype are an important resource for future studies on parasite factors associated with virulence or pathogenicity. The relative ease with which these parasites were generated offers the possibility of generating similar 'mutants' of *T. congolense* IL 1180 for comparative studies, and even increasing the proportion of such parasites by mutagenesis with classical mutagenic compounds.

**T2a.6 *Trypanosoma brucei* VSG mRNA binding proteins: the entire 141 nt 3'-region, including the end of the open reading frame of the VSG mRNA, is required for up-regulation of expression in bloodstream forms**

**Scientists:** R. Pellé, N. Murphy, P. Tebabi\*, L. Vanhamme\*, E. Pays\*

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Mechanisms for the transcriptional regulation of gene expression in trypanosomes are a strong focus of research in several laboratories. The recently developed UV-crosslinking technique to identify mRNA binding proteins (see *ILRAD 1993 Annual Scientific Report*) has encouraged a more detailed analysis of factors which influence VSG mRNA stability in the different life cycle stages of *T. b. brucei*. Previously, it has been shown that, in addition to the conserved 8-mer and 14-mer sequences found at the 3' end of VSG mRNA transcripts from *T. b. brucei*, other sequences at the 3' end are also involved in the formation of complexes between VSG mRNA transcripts and regulatory proteins in bloodstream form (BSF) parasites. In

recent analyses, RNA transcripts (31 nt long) corresponding to the 3' end of the VSG open reading frame (ORF) have been generated and used in the complex formation reactions. The results have shown that one RNA-protein complex is formed which differs from complexes observed using the entire NM8 RNA transcript of 141 nt. The formation of this complex is blocked by a DNA oligonucleotide which is complementary to this 3' ORF region. The combination of results strongly indicates that the entire 141 nt 3'-region of the VSG mRNA is involved in the binding of potential regulatory proteins that are likely to be involved in the regulation of VSG mRNA transcription.

Complementary studies carried out by Dr. E. Pays' group in Brussels have shown through transfection that the 141 nt 3'-region down regulates expression of a reporter gene in procyclic forms (PCF) and up-regulates it in BSF. UV-crosslinking experiments show specificity of binding of cytoplasmic proteins from BSF extracts, but not PCF extracts to transcripts containing the 141 nt 3'-region. Small deletions in the 141 nt 3'-region further support the conclusions of the UV-crosslinking experiments in that most of this region, including the end of the ORF, are contributing to this effect. Additional experiments with stable transfectants demonstrated that the influence is on the steady-state levels of mRNA, causing an increase in BSF and decrease in PCF steady-state levels. Mutations introduced into the 141 nt sequence suggest that this sequence is involved in several steps in the regulatory process which may also include processing, stability and translational efficiency. The results show that to achieve differentiation in trypanosomes, post-transcriptional control, and possibly translational control, is more important than transcriptional control. The identification and characterization of the proteins which specifically bind to the 3'-region of *T. b. brucei* VSG mRNA transcripts will help to further elucidate the mechanisms of gene control and differentiation in trypanosomes, and reagents generated from these studies can then be used to determine the generality of these control mechanisms for other differentially expressed genes and in other trypanosome species.

### **T2a.7 Identification and cloning of trypanosome cyclophilins using the RADES-PCR method**

**Scientists:** R. Pellé, N. Murphy, M. Sileghem

**Technician:** R. Thatthi

Increasing evidence suggests that African trypanosomes can modulate the immune system of their mammalian hosts by secreting molecules with the capacity to mimic or interfere with host cytokines. During the analysis of differentially amplified products generated by the RADES-PCR method (see *ILRAD 1993 Annual Scientific Report*) a 600 bp *Trypanosoma brucei brucei* product was isolated which has a four-fold higher level of expression in the actively-dividing long slender (LS) compared to the non-dividing short stumpy (SS) bloodstream forms (BSF). This product was sequenced and identified as a trypanosome cyclophilin homologue. Using this 600 bp fragment, a 1 kb and a 1.3 kb cDNA clone were isolated from *T. b. brucei* ILTat 1.1 and *T. congolense* IL 3000  $\lambda$ gt11 cDNA libraries, respectively.

Northern-blot analysis revealed a transcript of approximately 1.3 kb in both *T. b. brucei* and *T. congolense*. The level of the transcript in LS BSF is twice that of procyclic culture forms (PCF) and four-fold that of SS BSF. However, the transcripts appear to be expressed at a similar level in the four different life cycle stages of *T. congolense*.

Cyclophilins (Cyps) constitute a highly conserved family of proteins found in a wide variety of organisms. They have peptidyl-prolyl cis-trans isomerase activity, which catalyses the folding of proteins at proline-peptide bonds. Recently it has been reported that macrophages secrete Cyps in response to stimulation by lipopolysaccharides and that the cytokine, IL-8, is likely to be a Cyp variant. One possibility may be that Cyps are secreted by trypanosomes during the course of an infection to modulate immune responses of the mammalian host. Experiments to express the trypanosome Cyps in *Escherichia coli* are currently under way. If experiments demonstrate that trypanosomes secrete Cyps, it will be important to test the influence of these molecules on host lymphocytes.

### **T2b.1 Studies of T- and B-cell interactions in bovine immune responses**

**Scientists:** V. Lutje, K. Taylor

**Technician:** B. Gichuki

Previous studies have shown isotypic differences in the antibody response of susceptible and tolerant cattle breeds to a range of trypanosome antigens during infection with *Trypanosoma congolense*. To analyse the quality of T cell help provided to B-cells in health and infection, an assay has been established to evaluate the role of antigen-specific T cells in the development of antibody-secreting cells (ASC) *in vitro*.

T cell lines specific for *T. congolense* lysate (WTL) were established from immunized cattle. Lines were 85% CD4<sup>+</sup> and 15%  $\gamma\delta$ TCR<sup>+</sup>; they required autologous antigen-presenting cells (APC) and did not proliferate in response to an irrelevant antigen (ovalbumin). B-cells were obtained from peripheral blood mononuclear cells (PBM) by fluorescent-activated sorting of MA30<sup>+</sup> cells from cattle infected with *T. congolense*. Cultures comprising B-cells, APC and T cells were maintained with medium alone, WTL or pokeweed mitogen (PWM). The numbers of total antibody-secreting cells (ASC) were counted after six days in culture by an ELISpot assay (see *ILRAD 1993 Annual Scientific Report*). No ASC were detected in cultures maintained with medium alone. In the absence of T cells, no ASC developed in cultures stimulated with WTL. When WTL-specific T cells were added, the frequency of ASC was 25–35 per 10<sup>4</sup> cultured cells. In cultures stimulated with PWM, high numbers of ASC were detected. The addition of WTL-specific T cells did not affect the frequency of ASC.

Further studies are under way to determine (i) the frequency of antigen-specific ASC, (ii) the influence of T cell-derived cytokines on B-cell differentiation and (iii) the nature of ligand-receptor interactions that may affect development of ASC.

### **T2b.2 Localization of long-term cellular immune responses to *Trypanosoma congolense* in N'Dama cattle**

**Scientists:** V. Lutje, K. Taylor, E. Authié, A. Boulangé

**Technician:** D. Muteti

T cell mediated and antibody responses to whole lysate of *Trypanosoma congolense* (WTL), variable surface glycoprotein (VSG), congopain (CP) and hsp70/BiP homologue were analysed in a group of five N'Dama cattle that had previously experienced six infections with different clones of the parasite. The animals had been treated and remained aparasitaemic for more than three years.

The parameters of T cell-mediated responses measured were proliferation and IL-2 and IFN- $\gamma$  production. Proliferative responses of cells obtained from peripheral blood (PBM), spleen and lymph nodes (LN) were measured. Strong proliferation to WTL, VSG, CP and hsp70/BiP was detected in cells from lymph nodes but not in cells from spleen and peripheral blood. Cells from all sources mounted strong proliferative

responses to Concanavalin A (Con A). Levels of IL-2 were measured in supernatants of LN and spleen cells in a bioassay. Low IL-2 synthesis was detected in cultures stimulated with trypanosome antigens; slightly higher levels were detected in cultures stimulated with Con A. Levels of IFN- $\gamma$ , measured in LN cell cultures by a sandwich ELISA, were also low.

Antibody responses were measured in three animals. Antibodies to trypanosome antigens were detected in the sera of all three animals by Western blot analysis. Antibody-secreting cells (ASC) to trypanosome antigens were detected by an ELISpot technique. LN and splenic lymphocytes were cultured with recombinant bovine IL-2 (rboIL-2), lipopolysaccharide (LPS), VSG, WTL and the recall antigen foot-and-mouth virus (FMDV). After 6 and 14 days in culture the frequency of antibody-secreting cells (ASC) was measured. There was no increase in the frequency of ASC in LN or spleen cells after stimulation *in vitro* with VSG. However, after stimulation with WTL, increased numbers of ASC were observed in LN and spleen cell cultures from one animal (ND1) and from LN cultures of another animal, ND2. The same cell population responding to WTL also responded to FMDV and rboIL2. All cultures stimulated with LPS contained increased numbers of ASC.

Thus, LN cells, but not PBM or spleen cells, retain their capacity to proliferate to defined trypanosome antigens several years after infection. Their capacity to respond by IL-2 and IFN- $\gamma$  synthesis appears limited. However, although T cell proliferative responses to trypanosome antigens were only found in LN cells, splenic lymphocytes from one animal responded to activation with WTL by development of ASC. Interestingly, T cell proliferative responses did not correlate with differentiation of B-cells to ASC.

### **T2b.3 Cytokine gene expression in lymph node cells of Boran cattle infected with *Trypanosoma congolense***

**Scientists:** B. Mertens, V. Lutje, E. Authié, A. Boulangé

**Technicians:** K. Tikolo, Y. Verjee

It has previously been reported that trypanosome antigen-specific responses were detected in lymph nodes (LN) draining the site of infection (see *ILRAD 1993 Annual Scientific Report*). Proliferative responses could not be detected in peripheral blood lymphocytes (PBL). The highest proliferative responses were found in cultures of LN cells obtained 11 days post infection (p.i.). This time point was therefore chosen for an initial study on cytokine mRNA expression. Two Boran cattle (BL48, BL49) were infected with *T. congolense* IL 1180 and 11 days later the LN draining the chancre were removed.

Total RNA was extracted from freshly isolated LN cells (*ex vivo*), and from LN cells that were cultured for three days with VSG, congopain (CP), C-63 (a recombinant fragment of *T. congolense* hsp70/BiP protein), concanavalin A (Con A), or ovalbumin (OA). Cytokine profiles for two Th1 cytokines (IL-2 and IFN $\gamma$ ) and for one Th2 cytokine (IL-4) were determined by semi-quantitative RT-PCR. PCR prod-

ucts were visualized by ethidium bromide, gels were photographed and the negatives were scanned with a densitometer to allow a better estimation of the amounts of the PCR amplified products. IL-2 mRNA transcripts were very low or not detectable in cells of both animals *ex vivo*. Although high levels of IL-2 mRNA were detected in response to VSG in both animals, and to C-63 in one animal tested, IL-2 protein levels measured in culture supernatants were generally low. Con A and VSG induced IL-4 transcripts in both animals tested, the response to CP being much lower. In one of the animals, C-63 induced low level expression of IL-4. High levels of IFN $\gamma$  transcripts were detected *ex vivo*, 11 days p.i. The expression of IFN $\gamma$  increased after stimulation with VSG, CP and C-63, compared to the *ex vivo* and the Con A induced IFN $\gamma$  expression.

This cytokine pattern is suggestive of a Th0 type of response to defined trypanosome antigens. Th0 patterns are often described in early stages of parasitic infections. Cytokine analysis of LN cells later during infection could indicate if the helper T cell dichotomy Th1/Th2 also occurs during bovine trypanosome infections. The RT-PCR technique and a wider range of bovine specific cytokine primers, such as the Th2 cytokines IL-6 and IL-10, are available to address these questions in future studies.

#### **T2b.4 Effect of CD4 T cell depletion in *Trypanosoma congolense* infections**

**Scientists:** J. Naessens, M. Sileghem

**Research Fellow:** J. Buza

**Technicians:** J. Nthale, R. Saya, J. ole Gesharisha

Complete depletions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells have been achieved in blood, spleen and lymph nodes of cattle by intravenous injection of large amounts of mouse monoclonal antibodies to bovine CD4 or CD8 for at least two weeks. This technique was used to monitor the effect of CD4<sup>+</sup> T cells on the progress of disease (parasitaemia, anaemia and antibody responses) in *T. congolense* infections in Boran and trypanotolerant N'Dama cattle.

Four N'Damas and three Borans, all two years old and infected and treated with *T. congolense* clone IL 1180 one year previously, were used in the experiment. Two N'Damas and two Borans were depleted for CD4<sup>+</sup> T cells. The day after, all seven animals were infected with *T. congolense* clone 13E3 by five tsetse flies. Chancre formation at the bite site was greatly reduced in three of the depleted cattle and partially in the fourth. However, this did not affect the prepatent period and parasites appeared in the blood at the same time in CD4<sup>+</sup> T cell depleted and non-depleted animals and in both breeds.

CD4<sup>+</sup> T cells were absent from the blood for two weeks after depletion, but they reappeared to levels of 25% of those in the control, non-depleted animals. The IgG antibody response to trypanosome antigens in the control animals appeared five days after-infection, while this response was delayed in the depleted animals until after the second week and never reached the same levels as in the controls. However, in both breeds, parasitaemia and packed cell volume (PCV) were similar in depleted and non-depleted animals over the six weeks that they were monitored.

Previous studies have shown that the first peak of parasitaemia is slightly higher in Boran cattle than in the trypanotolerant N'Damas, and that PCV drops faster in Boran than in N'Damas. In this experiment we found similar differences between the two breeds around week 3. Within each breed, PCV and parasitaemia did not differ between depleted and non-depleted animals. Thus, total depletion of CD4<sup>+</sup> T cells for the first two weeks of the infection and partial depletion thereafter did not make the trypanotolerant N'Damas more susceptible. To prove that the differences in parasitaemia and PCV observed between the two breeds are not due to functional differences of CD4<sup>+</sup> T cells, the experiment will be repeated using naive N'Dama and Boran cattle and the total depletion of CD4<sup>+</sup> T cells will be extended for more than two weeks.

### **T2b.5 Development of methods to measure *Trypanosoma congolense* specific B-cell responses during infection**

**Research Associate:** K. Taylor

**Scientists:** D. Kennedy, P. Lessard, L. Logan-Henfrey

**Technician:** B. Gichuki

To determine whether the quantitative and qualitative differences between the serum antibody response in N'Dama and Boran cattle reflect the greater antigen load present in Boran cattle or are due to a more fundamental difference in B-cell function, an assay to measure, *ex vivo*, at the single-cell level, trypanosome antigen- and isotype-specific antibody responses has been developed.

We have previously reported an ELISpot assay (*ILRAD 1993 Annual Scientific Report*) in which the total number of antibody-secreting cells (ASC) can be measured. This method was further developed to measure the frequency of plasma cells secreting antigen-specific antibody of either the IgM or the IgG subclass. The frequency of ASC and antigen-specific ASC was compared between lymphocyte populations derived from peripheral blood, bone marrow, lymph node and spleen of *Trypanosoma congolense*-infected cattle. The number of ASC in each population was 50, 260, 1100 and 7870 secreting-cells per 100,000 lymphocytes, respectively. There were approximately equal numbers of cells secreting IgM and IgG from each population. Antigen-specific secreting cells were only detected in the spleen and their frequency was low, between 10 and 50 ASC per 100,000 lymphocytes. Attempts to improve the sensitivity of the assay by either depleting the lymphocyte population of T cells, thus increasing the frequency of B-cells in the population, or by increasing the total number of cells plated did not improve the sensitivity to a sufficient degree to detect antigen-specific ASC from lymphocytes collected from lymph node.

Having determined that the spleen is the best source of ASC, a biopsy procedure to sequentially sample spleen cells in adult cattle during the course of infection was developed using a 'TruCut' biopsy needle to collect core samples. Unlike spleen exteriorization, the spleen is surgically sampled *in situ*. This method is applicable to animals of any age or size and involves minimal trauma. The number of lymphocytes in a sample depends on the individual animal and on the general reactivity of the

spleen. In general, a single 20 mm spleen biopsy core contains approximately  $10^6$  lymphocytes. Several cores can be collected at a time. Splenic-lymphocytes can be used in an ELISpot assay to measure the frequency of cells secreting antigen-specific antibody of either the IgM or IgG subclass.

### **T2b.6 Determination of isotype and specificity of antibodies secreted by CD5<sup>+</sup> B lymphocytes in *Trypanosoma congolense*-infected cattle**

**Research Fellow:** J. Buza

**Scientist:** J. Naessens

Trypanosome infections in cattle are characterized by a concomitant increase in total number of B-cells, in the number of B-cells bearing the CD5 antigen and in serum IgM, some of which is reactive to non-trypanosome antigens. Since mouse and human CD5<sup>+</sup> B-cells (or B-1 cells) have been shown to produce polyreactive and autoreactive antibodies, an experiment was performed to determine whether the CD5<sup>+</sup> B-cells are the source of the non-trypanosome IgM observed in *T. congolense*-infected cattle. Ig-secreting B lymphocytes from spleen, but not peripheral blood, could be revealed by a silver-enhanced immunogold blot assay. Therefore, the spleen from an Ayrshire calf was exteriorized under the skin, so that spleen cells could be collected at regular intervals with a syringe. The animal was infected with *T. congolense* strain IL 1180 and splenic CD5<sup>+</sup> and CD5<sup>-</sup> B lymphocytes were sorted at weekly intervals to determine the frequency of cells producing antibodies (IgM and IgG) and cells secreting antibody specific for a non-trypanosome antigen, b-galactosidase. Unfortunately, due to technical difficulties, the blot assay could not detect cells producing antibodies to a whole trypanosome lysate. A lot of secreting B-cells were lost by the sorting procedure, as could be seen from the higher proportion of antibody secreting cells in unsorted spleen cells. Nevertheless, it was possible to compare the CD5<sup>+</sup> and CD5<sup>-</sup> sorted populations. Both B-cell populations contained the same proportions of IgG and IgM secreting cells. Cells secreting IgM antibodies to b-galactosidase and other non-trypanosome antigens were mainly detected in CD5<sup>+</sup> B-cells, although on one occasion in CD5<sup>-</sup> B-cells. Further analyses are required.

### **T2b.7 Analysis of phenotype and function of chancre-derived afferent lymph cells of cattle infected with *Trypanosoma congolense***

**Senior Research Fellow:** D. Mwangi

**Scientists:** D. McKeever, D. Williams\*, J. Naessens, D. Kennedy, S. Moloo

**Research Associates:** K. Taylor, J. Magundu

**Technicians:** P. Mucheru; B. Gichuki, J. Kabata

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The initial responses of susceptible mammalian hosts to infections with *Trypanosoma congolense* include the development of localized skin reactions (chancres) at sites of

infected tsetse fly bites. The chancre is thought to be important in both the pathogenesis of the disease and induction of serodeme-specific protective immune responses. Previous studies of the chancre focused on aspects of trypanosome-induced immunosuppression. Trypanosomes were not detected in afferent lymph during these studies. However, the phenotype and some functions of chancre-derived afferent lymph cells were modulated. Although a proportion of these cells expressed activation markers their responsiveness to Concanavalin A (Con A) was suppressed. These studies have been extended by examining the general induction of the early immune responses of cattle to *T. congolense* infection. Specifically, the parasite dynamics, phenotypes and functions of chancre-derived afferent lymph activated cell populations were monitored.

Pseudo-afferent lymphatic ducts of Boran calves were cannulated. Animals were infected with tsetse-transmitted *T. congolense* IL 1180 within the drainage area of the cannulated lymphatic. Detectable chancres developed from six days after infection. Trypanosomes were detected in afferent lymph seven days post-infection (dpi) with peak parasitosis occurring 9-11 dpi when up to  $7.5 \times 10^7$  trypanosomes left the chancre every 24 hours. Thereafter the number of parasites in lymph fluctuated but gradually declined. Immunization of calves with whole trypanosome lysates prior to cannulation and infection did not affect the prepatent period, though the parasitosis was generally lower.

Concurrent with the appearance of trypanosomes in afferent lymph, an increase in total cell output, primarily T cells (CD4<sup>+</sup>, CD8<sup>+</sup> and  $\gamma\delta$  T cells) and to a lesser extent afferent lymph veiled cells (ALVCs), was observed. The proportions of these cells either remained at pre-infection levels or increased only slightly. An increase in proportion and total output of B-cells occurred from 14 dpi. Increase in total cell output was accompanied by increases in proportions and absolute output of cells expressing T cell activation markers; interleukin 2-receptor  $\alpha$  chain (CD25), and activation antigen 1 (ACT1). These activated T cells were mainly of the CD4<sup>+</sup> phenotype. Similarly, the proportions and output of cells expressing the activation antigen, transferrin receptor (CD71), which were mainly B-cells and ALVCs, also increased.

An ELISpot assay was used to determine the frequency of antibody secreting B-cells (*ex vivo*) in chancre-derived afferent lymph. Antibody secreting cells (ASCs) were only detected from 16 dpi at a maximum frequency of one percent (1%). However, no trypanosome specific ASCs were detected at any time when plates were coated with whole trypanosome lysates. It is probable that the frequency of such cells in afferent lymph is below the detection level of this assay. Using ELISA, trypanosome-specific antibodies were detected in lymph plasma from 14 dpi. The antibodies were mainly of the IgM isotype. The presence of ASCs and specific antibodies corresponded well with the increase in proportion and output of B-cells in afferent lymph.

Proliferative responses of chancre-derived afferent lymph cells to Con A and foot-and-mouth disease virus were abrogated from 5 dpi. Similarly, in animals previously immunized with whole trypanosome lysates (WTL), the response to WTL was suppressed. Changes in cytokine profiles of afferent lymph cells collected during the development and regression of the chancre were also noted (see Abstract T2b.8).

These studies show that in cattle there is intense proliferation of trypanosomes in the chancre which migrate to the draining lymph node. In addition, the phenotypes and function of cells migrating from the chancre are modulated. These cells are activated and elaborate specific antibodies and cytokines which may be of significance both in immune responses and pathogenesis of the disease.

### **T2b.8 Cytokine gene expression in bovine afferent lymph cells during infection with *Trypanosoma congolense***

**Scientists:** B. Mertens, D. Mwangi, D. McKeever

**Technicians:** K. Tikolo, Y. Verjee

Early events in the inflammatory and immune response to *T. congolense* infection in cattle are under investigation. Afferent lymph cells from a Boran calf (BL21) were collected during primary infection with *T. congolense* at various time points (day 1 preinfection(-1); days +1, 2, 8, 9, 14 to 23, 25 post-infection (p.i.). Semi-quantitative RT-PCR was used to determine whether a particular cytokine mRNA was expressed. PCR products were visualized by ethidium bromide staining. Results can be summarized as follows: (i) cytokines with low levels of expression: IL-2—weak signal on day -1, undetectable all the other days except for a weak signal at days 19, 20, 21 and a more intense signal on day 25. IL-4—very weak signal on all days. (ii) cytokine expression at all time points: TNF $\alpha$  and TGF $\beta$ —both showed very strong signals. (iii) cytokines with fluctuating levels of expression: IFN $\gamma$ —appears at day 2 p.i., remained high during the infection, with a peak on days 19, 20, 21. IL-1 $\beta$ —strong signal on days -1, +1, 2 followed by a lower expression until days 19, 20, 21 where a more intense signal was observed. IL-10—strongest on days -1, 2, 20. On days 2, 14 to 19 and 23 p.i. the level remained high, the following days the signal was weaker. GM-CSF, present on days -1, +1, 2, then the signal became weaker, but remained detectable until a peak at day 20, and subsequently fell. CD40 Ligand (CD40L)—peak on days 19, 20, 21, with the expression otherwise remaining medium to low.

With these preliminary data it is too early to speculate in detail on the significance of these cytokine profiles, but by comparison with some data reported from other species some comments can be made. In mice and humans, TNF- $\alpha$ , IL-1 $\beta$  and GM-CSF have been shown to contribute to the maturation and migration of dendritic cells. TNF $\alpha$  and IL-10 transcripts have been detected in normal mouse skin. These cytokines were also present in the bovine preinfection samples. It was reported in mice that IL-10 and TGF $\beta$ , when produced in concert during T cell dependent anti-parasitic responses, have a potent down-regulatory effect on host macrophage effector activity and production of Th1 cytokines (IL-2, IFN $\gamma$ ) and thereby may prevent IFN $\gamma$  from being fully effective.

Phenotypic analysis (see Abstract T2b.7) of the afferent lymph cells indicates there is an increase in IL-2R from day 7 onwards, although transcripts for IL-2 become undetectable from preinfection until day 19 p.i. The significance of this observation is not yet known, but the upregulation of several cytokines at days 19 to 20 seems

significant, but it is not yet possible to correlate these findings with other parameters. An increase in B-cells and detection of IgM immunoglobulins was observed from day 14 p.i. Immunoglobulins of the IgG isotype were present from day 19 p.i. onwards. This observation is consistent with the upregulation of CD40L from day 19 since this factor plays an essential role in Ig isotype switch in humans and mice (see Abstract T2b.14).

### **T2b.9 Antigen presentation by afferent lymph veiled cells (ALVC) during bovine trypanosome infection**

**Scientists:** V. Lutje, D. Mwangi, D. McKeever

Bovine afferent lymph veiled cells (ALVC) have been shown to be potent antigen presenting cells (APC) for antigen-specific T cells and to be able to induce primary immune responses in naive T cells. ALVC from local skin reactions which develop at the initial site of trypanosome infection have been investigated to determine (i) if they can efficiently present trypanosome antigens to primed T cells, (ii) how APC function, and (iii), subsequent immune responses occurring in the draining lymph node during trypanosome infection. T cell lines specific for whole trypanosome lysate (WTL) were developed from PBM obtained from immunized animals. T cell lines were 85% CD4<sup>+</sup> and 15%  $\gamma\delta$  TCR<sup>+</sup> (see Abstract T2b.1).

The prescapular lymph nodes were subsequently removed from these animals and afferent lymph was collected by cannulation of pseudo-afferent lymphatic ducts. Animals were infected with *Trypanosoma congolense* by tsetse bites in the drainage area of the pseudo-afferent lymphatic ducts. ALVC were prepared from afferent lymph by flotation on 75% Ficoll-Paque density gradients in Ca<sup>2+</sup>, Mg<sup>2+</sup> free HBSS.

The output of ALVC in afferent lymph was measured at various time points post-infection (p.i.). Concomitant with an increase in the total cell output in afferent lymph draining the site of infection was an increase in the number of ALVC from 9 days p.i., from  $1 \times 10^6$ /ml pre-infection to  $10\text{--}20 \times 10^6$ /ml at 14 days p.i. Cultures were established comprising  $5 \times 10^4$  WTL-specific T cells/well and irradiated ALVC at concentrations from  $2 \times 10^4$  to  $2.5 \times 10^3$ /well. WTL was added at a final concentration of 20 mg/ml. Proliferative responses were measured after three days. No proliferation was detected in response to an unrelated antigen (ovalbumin) or in the absence of APC. No major differences in proliferative responses were detected between cultures that had received different concentrations of ALVC.

An increase in proliferative responses to WTL was observed when ALVC collected on day 3 p.i. were used as APC; responses using ALVC collected on days 7 and 10 were comparable to pre-infection values. ALVC isolated from lymph up to day 10 p.i. were used in this study, but technical problems prevented the necessary extension of the cannulations to determine the extent of changes in APC function by ALVC during trypanosome infection.

**T2b.10 Expression of partial and full length recombinant BiP-homologue (69 kDa antigen)****Research Fellow:** A. Boulangé**Scientist:** E. Authié**Technician:** D. Muteti

The gene encoding a major trypanosome antigen recognized by *T. congolense*-infected cattle was cloned and sequenced (*ILRAD 1993 Annual Scientific Report*, T2a.1). The protein belongs to the hsp70 family and is homologous to the mammalian immunoglobulin heavy chain binding protein (BiP). Two partial recombinant proteins, C-28 and C-63, were expressed in *Escherichia coli* and were used to study immune responses in N'Dama and Boran cattle (*ILRAD 1993 Annual Scientific Report*, T2b.6). To further assess possible differences in the nature of epitopes recognized by the two breeds of cattle, three more recombinant fragments of the BiP homologue have been expressed using the pMAL expression system. N-14 is encoded by an *EcoRI-PstI* fragment of the ORF and corresponds to the first 14 kDa at the N-terminus. N-61, from an *EcoRI-HindIII* fragment, corresponds to 61 kDa on the N-terminal side. C-9, from an *XhoI-BamHI* fragment, is a 9 kDa polypeptide containing the non-conserved C-terminal sequence of the molecule. The respective fusion proteins were cleaved from the maltose binding protein and recombinant proteins were prepared, with the exception of N-14 which underwent rapid degradation after cleavage. None of the monoclonal (MAB) or polyclonal antibodies raised against native 69 kDa protein reacted with the N-14 protein. Only one of the three MABs reacted with N-61. All available antibodies, with the exception of the MAB reacting with N-61, reacted with C-9, confirming that the C-terminal region of the molecule contains the majority of the B-cell epitopes.

Using the polymerase chain reaction, a complete ORF bearing adequate restriction sites (*EcoRI-BamHI*) for cloning into pMAL had been synthesized. However, due to instability of the DNA fragment in plasmid vectors, expression of the full length 69 kDa recombinant protein had remained unsuccessful. After repeated attempts using various bacterial strains, successful propagation without artifactual recombinations was finally obtained *in vivo* in the strain SURE-2 (Stratagene). The expressed 111 kDa fusion protein could be purified and subsequently cleaved to generate the 69 kDa recombinant BiP-homologue.

**T2b.11 Expression in *Escherichia coli* of the central and C-terminal domains of a *Trypanosoma congolense* cysteine protease (congopain)****Scientist:** E. Authié**Research Fellow:** A. Boulangé**Technician:** D. Muteti

The sequence of a *Trypanosoma congolense* cysteine protease, as reported by Fish *et al.* (*ILRAD 1993 Annual Scientific Report*, T2a.6) presents an hydrophobic N-terminal leader sequence and a propeptide which are processed to generate the mature

protease. The mature protease is composed of two major regions. A central domain (CD, 23 kDa) containing the catalytic site is homologous to papain and highly conserved in evolution amongst different species. CD is linked by a polyproline hinge to an unusual C-terminal domain (CTD, 11 kDa) which is only present in cysteine proteases of trypanosomatids.

CTD and CD have been expressed independently in *E. coli* using the pMAL vector expression system. Two sets of oligonucleotides were designed for use in the polymerase chain reaction. One set is homologous to the 5' end of each open reading frame (ORF) and introduces an *EcoRI* site before the first codon; the other set is homologous to each 3'-end and introduces a *HindIII* site. In addition, the 3'-end oligonucleotide of the CD ORF includes a stop codon before the *HindIII* cloning site. This was designed to allow unidirectional cloning of each ORF into pMAL.

CD and CTD were produced as fusion proteins of 77 and 50 kDa, respectively, which were predominantly insoluble and present in inclusion bodies. The yields were 100 to 200 mg per litre of culture. A small amount of soluble CTD fusion protein could be purified on an amylose column and the recombinant CTD was prepared according to the standard procedures for the pMAL system. Inclusion bodies of the CD fusion protein were purified and solubilized in 10 M urea. Following solubilization, the CD fusion protein underwent spontaneous cleavage, resulting in the generation of a fragment with an apparent MW of 26 kDa. N-terminal amino acid sequencing of this fragment confirmed its identity to CD. Protease inhibitor profiles indicated that the cleavage of the CD fusion protein is probably due to a bacterial metallo-proteinase. The 26 kDa recombinant CD was purified by anion exchange chromatography and partially solubilized. However, attempts to fully renature the recombinant CD in order to produce active enzyme have so far been unsuccessful. This may be due to the presence in CD of seven cysteine residues, six of which are involved in forming disulfide bridges. Eukaryotic expression systems, such as the baculovirus system, are currently being considered for expression of the CD.

## **T2b.12 Antigenicity of the central and C-terminal domains of congopain**

**Scientist:** E. Authié

**Research Fellow:** A. Boulangé

**Technician:** D. Muteti

Two recombinant fragments of a *T. congolense* cysteine protease (Fish *et al.*, *ILRAD 1993 Annual Scientific Report*), representing the catalytic, central domain (CD) and the C-terminal extension (CTD), were expressed in *Escherichia coli* (see Abstract T2b.11). A monoclonal antibody raised against native congopain reacted with CTD, the non-conserved region of the cysteine protease, thus confirming the identity between congopain and the cysteine protease described by Fish *et al.* The sera from mice and rabbits immunized with native congopain reacted on Western blots with CTD, but did not react with CD. Screening of sera from *T. congolense*-infected cattle confirmed that CTD is a major target of antibody responses. However, despite its high degree of conservation (and unlike the CD of cruzipain

in *T. cruzi*-infected people), CD may be antigenic in *T. congolense*-infected cattle. Two N'Dama and two Boran cattle which had similar levels of antibody to native congopain after multiple infections with *T. congolense* were tested for serum reactivity to CD. The sera from the two infected N'Damas, but not those from the two infected Borans, reacted with CD on Western blots. Thus, following infection with *T. congolense*, N'Dama cattle may recognize epitopes associated with the catalytic site of congopain. This observation is in agreement with previous indications that there may be differences in the nature of epitopes recognized by trypano-tolerant and trypanosusceptible cattle. Following immunization with recombinant CD, both N'Dama and Boran cattle developed high levels of anti-congopain antibodies, as assessed by Western blotting and ELISA. However, these antibodies did not react with native congopain and showed no effect on the proteolytic activity of the enzyme.

### **T2b.13 A monoclonal antibody to bovine $\alpha$ IIb $\beta$ 3-integrin (BoCD41)**

**Scientists:** J. Naessens, J. Syfrig

**Technician:** J. Nthale

A monoclonal antibody, IL-A164, was obtained that was specific for bovine platelets and a subset of bone marrow cells. It precipitated, from bone marrow cells and platelets, a molecule composed of two chains of approximate Mr 130 and 92 kDa under reducing, and Mr 140 and 80 kDa under non-reducing conditions. Endoglycosidase-F treatment showed that both polypeptide chains contained approximately 10% N-linked carbohydrate. Although the light chain has the same Mr as the bovine  $\beta$ 2-integrin, preabsorption with an antibody to the bovine  $\beta$ 2-light chain (obtained from Prof. Letesson, Namur, Belgium) did not remove the antigen of IL-A164. Large amounts of the antigen were prepared by affinity-purification with immobilized IL-A164 and sent to Prof. T. Pearson, Victoria, Canada, for an amino-terminal sequence analysis. Over 20 amino acids could be sequenced from both polypeptide chains and showed the highest homology (over 70%) with human gpIIIa and gpIIb. Thus MAb IL-A164 recognizes the bovine homologue of human  $\alpha$ IIb $\beta$ 3-integrin, also called CD41.

### **T2b.14 Cloning of bovine CD40 Ligand**

**Scientist:** B. Mertens

**Research Associate:** E. Gobright

**Technicians:** C. Muriuki, Y. Verjee

Studies of antibody responses in *T. congolense*-infected cattle to variant and invariant trypanosome antigens have suggested an impairment in the switch from IgM to IgG<sub>1</sub> in trypanosusceptible cattle during infection. Isotype switching involves T lymphocyte help and a close interaction between T and B-cells through

a series of surface markers on both cell types. One of these surface markers, expressed on activated T cells, is known as CD40 Ligand (CD40L, also referred to as gp39, TRAP or T-BAM).

Murine and human cDNAs coding for CD40L have been recently cloned. To isolate the bovine analogue, primers were designed based on interspecies homologies. Total RNA was extracted from concanavalin A-stimulated peripheral blood lymphocytes and reverse transcribed into cDNA. Using the primers, DNA fragments were amplified by PCR, subcloned, and characterized by sequencing. Based on homology comparisons, a bovine cDNA coding for bovine CD40L was identified. The bovine clone is 750 nt in length, encoding a polypeptide of 245 amino acids (aa). Bovine and human CD40L are highly homologous. Within the coding region they share 88% identity at the nt level, and 89% at the aa level. Similar to the human CD40L, the bovine polypeptide has the characteristics of a type II membrane protein, with an N-terminal intracellular domain, of which a few aa are lacking in the bovine clone due to the cloning strategy used. This region is followed by 24 predominantly hydrophobic residues consistent with a transmembrane domain and 215 aa constituting the putative extracellular carboxy-terminal domain.

Comparisons of human and mouse CD40L aa sequences with those of other published sequences revealed significant similarities to TNF $\alpha$  and TNF $\beta$ . Structural alignment suggests that the aa sequence of human CD40L is compatible with the TNF $\alpha$ -like folding. Similar homologies are present when comparing the aa sequences of boCD40L and boTNF $\alpha$ .

Work is in progress to express bovine CD40L in COS cells. The availability of recombinant boCD40L will enable investigations of B-cell differentiation and immunoglobulin isotype switching in relation to African trypanosomiasis in cattle.

### **T2b.15 Identification, purification and partial characterization of a molecular chaperone associated protein from *Trypanosoma brucei* localized within the endoplasmic reticulum**

**Scientists:** D. Nandan, T. Pearson\*

**Research Associate:** C. Wells

**Technician:** D. Ndegwa

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Molecular chaperone molecules can bind to denatured or misfolded proteins within cells. This property was utilized to separate them from total cell lysates of *T. b. brucei* using a gelatin binding/ATP release assay. The released proteins were separated electrophoretically and antisera raised against each. An antiserum to a 72 kDa gelatin bound, ATP-released protein was then used to isolate associated proteins from solubilized membranes of *T. b. brucei* bloodstream forms (BSF) by immunoaffinity chromatography. A 44 kDa chaperone-associated protein proved to be immunogenic and the antiserum raised against the 44 kDa protein was further used to purify,

characterize and localize the protein. Total solubilized membranes from *T. b. brucei* BSF and procyclic forms, when probed with the anti-44 kDa serum, both showed the protein present as a 44/45 kDa doublet. Similar blotting experiments with other trypanosome antigens showed reactivity of a single band of 53 kDa in all life cycle stages of *T. congolense* and a doublet of 53/56 kDa in *T. vivax* BSF. No cross reactivity was observed with total cell lysates from mammalian host species.

Indirect immunofluorescence using affinity purified antisera specific for the 44 kDa protein showed labelling of the perinuclear area and the reticular system extending throughout the cell in all three species of trypanosome examined. Immunoelectron microscopy revealed these structures to be the perinuclear and endoplasmic reticulum (ER). The labelling was restricted to these sites and found nowhere else in the trypanosomes. Protease protection experiments demonstrated that the epitopes bound by the antisera are buried within the membrane or towards the luminal face of the ER. The function of this protein(s) remains unknown. However, ruthenium red overlay of nitrocellulose blots containing the 44/45 kDa doublet showed strong binding of the dye to the doublet and the binding was specifically inhibited by 50 mM CaCl<sub>2</sub>. This suggests that the protein(s) have a potential to bind calcium.

In other eukaryotic cells some proteins within the endoplasmic reticulum play an important role in maintenance of calcium homeostasis and there are reports that the endoplasmic reticulum of trypanosomes performs a similar function. It is possible that the 44 kDa protein may fulfill a role in calcium homeostasis, intracellular signalling or protein folding. It may also serve as a useful marker of the endoplasmic reticulum membrane for biochemical studies.

**T2c.1 Destruction of erythrocytes by macrophages *in vitro*:  
lack of correlation with trypanotolerance****Senior Research Fellow:** A. Makumyaviri**Scientist:** M. Sileghem**Technicians:** R. Saya, P. Mucheru

Uptake and destruction of erythrocytes by activated macrophages is considered an important factor in the development of anaemia during bovine trypanosomiasis. Preliminary studies in which macrophages isolated from infected animals were cocultured with  $^{51}\text{Cr}$ -labelled erythrocytes revealed an increase in both uptake and release of  $^{51}\text{Cr}$  by the macrophages during infection. It was then suggested that both parameters could be used *in vitro* as an indicator of macrophage activation, representing phagocytosis and intracellular destruction respectively. Since the increase in these parameters during infection was much higher in trypanosusceptible cattle than in trypanotolerant ones, differential macrophage activation in both breeds was considered a possible cause of the different disease susceptibility.

In a previous study  $^{51}\text{Cr}$  has been shown to be an unreliable indicator of macrophage activation. Despite different experimental conditions, the observed release was usually not markedly higher than the spontaneous release and no differences were observed between monocytes from normal animals and monocytes from trypanosome-infected animals. However, uptake of either  $^{51}\text{Cr}$ - or fluorescein-labelled erythrocytes appeared to be a useful method for analysis of erythrophagocytosis. Controls in which the monocytes were separated from the erythrocytes by a semi-permeable membrane were routinely included to assess the level of background uptake of the label. In contrast to the  $^{51}\text{Cr}$  release, uptake of  $^{51}\text{Cr}$  was found to be increased as a consequence of trypanosomal infections. The same method has been used to study possible differences between tolerant and susceptible cattle. Both Boran and N'Dama cattle were infected with *T. congolense* IL 1180 by tsetse fly transfer. At different time intervals throughout infection, monocytes were purified and cocultured with fluorescein- and  $^{51}\text{Cr}$ -marked autologous erythrocytes *in vitro*. As noted previously, a modest increase was observed, but no differences were seen between the two breeds. Introduction of opsonizing agents throughout the experiment did not affect this outcome.

**T2c.2 Production and assay of IL-1 $\alpha$  and IL-1 $\beta$   
during trypanosomiasis in cattle****Scientist:** M. Sileghem**Research Associate:** L. Gaidulis

Using the thymocyte costimulation assay, it has been reported previously that production of interleukin 1 (IL-1) and related cytokines is higher in the tolerant N'Dama breed of cattle than in the susceptible Boran breed during the early stages of infection with *T. congolense* IL 1180 (one to two weeks after infection). Since IL-1

is mainly produced by cells from the monocyte/macrophage lineage, this suggested a possible correlation between macrophage activation and trypanotolerance. However, analysing the production of another macrophage-derived cytokine, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), in Boran cattle infected with either *T. vivax* IL 2337 (which causes a severe acute anaemia) or *T. congolense* IL 1180 (which causes a more gradual drop in packed cell volume), a possible correlation between severity of disease and macrophage activation was observed. Since the TNF- $\alpha$  titres produced during *T. congolense* infection were too low to be titrated with the immunological detection assay, it was not possible to compare tolerant with susceptible animals during *T. congolense* infection.

To address this deficiency, an assay to detect activation of IL-1 $\alpha$ , IL-1 $\beta$  and TNF- $\alpha$  at the mRNA level has been developed. By polymerase chain reaction (PCR) it was possible to reduce the limit of detection considerably so that it became feasible to analyse samples which could not be analysed with the protein-detection systems. Peripheral blood samples were collected weekly from four Boran cattle infected with *T. vivax* IL 2337, four Boran cattle infected with *T. congolense* IL 1180 and four N'Dama cattle infected with *T. congolense* IL 1180. All animals were sampled up to four weeks after infection. Total RNA was analysed from both freshly isolated monocytes and from cultured monocytes. The latter samples were included to assess the impact of cultivation on the activation stage as all immunological titrations had been performed *ex vivo* with cultured monocytes. The RNA was reverse transcribed into ss cDNA and used as a template in the polymerase chain reaction with IL-1 $\alpha$  and IL-1 $\beta$  specific primers derived from published sequences or TNF- $\alpha$ -specific primers derived from sequencing at ILRAD. The results were analysed after electrophoresis of the PCR products through an ethidium bromide stained agarose gel.

IL-1 $\alpha$  and especially IL-1 $\beta$  were expressed constitutively at low levels prior to infection, with the cultured monocytes showing much higher pre-infection levels. IL-1 $\alpha$  increased early in both breeds following infection with both trypanosome species (week 1 p.i.) whereas IL-1 $\beta$  levels appeared to increase in most animals during week 2 p.i. TNF- $\alpha$  increased early in infection during both *T. congolense* and *T. vivax* infection, but the increase was considerably stronger during the latter, confirming earlier observations. Differences between Boran and N'Dama cattle during *T. congolense* infection were too subtle to be picked up by the used PCR methodology for all three cytokines. Therefore, it will be critical to establish a quantitative method to compare cytokine levels in both breeds (See Abstract T2c.3).

### **T2c.3 Standardization of an assay for quantification of bovine cytokine mRNA levels**

**Research Associate:** L. Gaidulis  
**Scientist:** M. Sileghem

To date, polymerase chain reaction amplification (PCR) has proved the only method which was sensitive enough to titrate cytokines in experimental samples from

trypanosome-infected cattle but it did not allow quantitation of the levels for routine cytokine analysis.

Several approaches for message quantification were considered, all based on a careful Standardization of the PCR prior to detection and quantification. The commercially available QUANT AMP ASSAY (Amersham) was employed to test the methodology for bovine cytokines. In this system, RNA is reverse transcribed into ss cDNA and used as a template in a carefully standardized PCR with gene-specific primers and tritiated deoxynucleotides. The resulting amplified tritiated product was then hybridized with a biotinylated internal oligonucleotide in an aqueous environment. The hybrid is captured on streptavidin-coated scintillation fluoromicrospheres. Quantification is achieved by scintillation counting and the incubation in the assay of an internal standard such as  $\beta$ -actin RNA.

Quantification is based on the assumption that there is a linear relationship between the quantity of mRNA and the final PCR product provided that there is a faithful conversion of the test sample to cDNA and exponential amplification. But it is well known that the PCR reaction after a certain number of cycles—which vary for the different primer sets and genes analysed—often enters a plateau phase where this relationship is no longer valid. Therefore, it was necessary to optimize the PCR cycles for each primer set to be used. Oligonucleotides for TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$  and two housekeeping genes,  $\beta$ -actin and GAPDH, as well as internal biotinylated capture oligonucleotides, were designed and tested on lipopolysaccharide (LPS) stimulated macrophages and on freshly collected and unstimulated macrophages. Various cDNA dilutions and amplification cycles were tried out to determine the range in which a linear relation was observed. Amplification during the exponential phase occurred between 20–23 cycles for bovine  $\beta$ -actin, but after further cycles for the other cytokines. Similarly, dilution of the cDNA by 1:4 to 1:8 produced clear differences in the quantity of IL-1 $\alpha$  mRNA expression in RNA samples which previously had been classified as positive. This method is being utilized to titrate IL-1 $\alpha$ , IL-1 $\beta$  and TNF- $\alpha$  in selected, conserved experimental samples to relate cytokine levels to previously observed changes in immunological parameters during infection.

#### **T2c.4 Role of the glycosylphosphatidylinositol anchor of the variable surface glycoprotein from African trypanosomes in macrophage activation**

**Scientists:** M. Sileghem, J. Naessens

**Technician:** R. Saya

It has been previously shown that production of the macrophage-derived cytokine, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), is induced during African bovine trypanosomiasis and that the level of production appears to be associated with severity of disease. In the present study, the identity of parasite molecules responsible for this macrophage activation has been investigated. The identification of such parasite 'toxins' is a field of growing interest related to anti-disease intervention. In malaria, the glycosylphosphatidylinositol (GPI) anchor of *Plasmodium falciparum*

surface proteins has been shown to cause macrophage activation and cleavage of the *sn*-1,2-diacylglycerol, which represents the actual membrane anchor, resulted in a complete loss of TNF- $\alpha$  induction.

Preliminary results have shown the variable surface glycoprotein (VSG) of trypanosomes to be a strong inducer of TNF- $\alpha$  production and subsequent experiments have focused on a putative involvement of the anchor.  $^3\text{H}$ -myristic acid was used to label the *sn*-1,2-dimyristyl glycerol anchor of *Trypanosoma brucei brucei* 221 VSG which was purified in its membrane associated form by a routine biochemical purification based upon water:methanol:chloroform extraction. Following gel electrophoresis and autoradiography, a strong incorporation of the label in the VSG was observed; contaminating proteins were present in the preparations but were unlabelled. When treated with phosphatidylinositol-specific phospholipase C, up to 90% of the dimyristylglycerol could be cleaved.

When the untreated and phospholipase C-treated VSG samples were added to primed bovine monocytes, TNF- $\alpha$  induction was observed with both fractions. Although these studies argued against a specific involvement of the anchor, other interpretations were possible. The possible involvement of contaminating bacterial endotoxins was ruled out by the demonstration that polymyxin B effectively blocked stimulation by bacterial lipopolysaccharide (LPS) but not stimulation by trypanosomal VSG. The possibility that the cleaved dimyristylglycerol could still interact with the cells was studied by extraction of the whole sample with methanol:hexane. Despite a considerable loss of biological activity, both the uncleaved and the cleaved preparations were stimulatory. Finally, further purifications were attempted to study the involvement of contaminating proteins. Following unsuccessful attempts to separate the VSG from a dominant 70 kDa contaminant by chromatography, preparative electrophoresis was tested. Control VSG samples run in a sodium dodecyl sulphate (SDS) polyacrylamide gel under reducing conditions were found to retain their biological activity following partial removal of the SDS. However, trace amounts of detergent could interfere with the phospholipase C treatment. Therefore, the samples were first treated with phospholipase C and subsequently purified. Both samples were found to be able to trigger TNF- $\alpha$  production.

In all the above studies, monocytes were used which were primed overnight with IFN $\gamma$ . This treatment does not induce an effector function but makes the cells hyper-responsive to external triggers such as bacterial LPS. When bovine monocytes are kept in a resting state by cultivation overnight in culture medium, little, if any, response is seen. In this resting state, monocytes are still triggered to produce TNF- $\alpha$  by the VSG containing the anchor but no longer by the VSG from which the anchor has been cleaved. Thus, in contrast to the situation in *Plasmodium*, the anchor of the VSG is not the actual TNF- $\alpha$  trigger but plays an important accessory role in macrophage activation.

## **T2c.5 Modifications in the production and purification of biologically active bovine tumour necrosis factor- $\alpha$**

**Research Associate:** L. Gaidulis

**Scientist:** M. Sileghem

**Visiting Research Fellow:** M. Lem

The role of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) in trypanosomiasis is controversial and neutralization of its effect *in vivo* is an approach to clarify the action of this cytokine. Two different TNF receptors have been characterized and non-receptor-mediated biological functions have been demonstrated. As a consequence, efficient overall neutralization *in vivo* appears to require polyclonal antisera. The expression of biologically active bovine recombinant TNF- $\alpha$  has been previously reported. In this report the scaling up of the expression procedure to produce a large batch of active recombinant protein is described to provide material for production of polyclonal antisera.

Expression of bovine TNF- $\alpha$  has been achieved using the pMAL-P vector in which the cloned gene is fused to a gene coding for a maltose-binding protein (MBP). Following induction in *Escherichia coli*, the fusionprotein (FP) was purified from bacterial products by affinity chromatography on an amylose resin. This procedure yielded 7–12 mg of FP protein from 1 litre transformed *E. coli*. While the resulting FP was 90–95% pure, about 1–2 mg/ml were lost following concentration and dialysis. By changing the growth medium and providing more aeration during the induction phase, the FP yield was increased to 20–40 mg/l of culture. One of the major problems which was encountered previously was the incomplete cleavage of the FP with the site-specific enzyme, Factor Xa protease, yielding a mixture of uncut FP, the carrier protein MBP and the 17 kDa recombinant TNF- $\alpha$ . The efficiency of cleavage was considerably increased by addition of 0.5 M Urea.

Several methods were tried for removing the carrier and the small amount of remaining FP. Methods tested included affinity chromatography, HPLC anion exchange chromatography using a Pharmacia MonoQ 5/5 column, and size exclusion chromatography using a Pharmacia Superose 12 column. Following elution in a 0–0.5 M NaCl gradient in the anion exchange purification, the MBP and TNF- $\alpha$  co-migrated consistently. Furthermore, despite clear differences in molecular weight between TNF- $\alpha$  and MBP observed in gel electrophoresis, the spontaneous formation of trimers by the recombinant TNF- $\alpha$  resulted in a size exclusion volume similar to the MBP. Therefore, gel electrophoresis, which consistently showed good resolution was used to purify the protein on a preparative scale. Following electroelution, 500 mg of TNF- $\alpha$  was purified. The uncleaved fusion protein, the cleaved mixture containing MBP and TNF- $\alpha$  and finally the electroeluted pure TNF- $\alpha$  were all confirmed to be biologically active by titration on WEHI 164 clones 13 cells.

**T2d.1 Preinfection status of bone marrow in cattle****Scientists:** A. Andrianarivo, L. Logan-Henfrey**Technician:** P. Muiya

In a retrospective analysis, the preinfection status of the bone marrow has been examined in two groups of cattle separately infected with *Trypanosoma congolense*. The first group included six adult cattle (4–5 years of age) with three N'Damas and three Borans. The second group involved eight yearling cattle (12–16 months of age) with four N'Damas and four Borans. Sternal bone marrow samples were collected at weekly intervals for three weeks prior to the infection. Aliquots of the bone marrow aspirates were diluted in Turk's medium containing 1% acetic acid (to remove mature red blood cells) and the total nucleated cells (TNC) were counted. The TNC, expressed per ml of bone marrow, were similar in N'Damas and Borans in the two age groups of cattle. However, the numbers were approximately 1.5 times higher in yearling cattle when compared to adult cattle. Bone marrow mononuclear cells (BMMNC) were obtained following two sequential Ficoll-Paque density centrifugations and the yields in BMMNC per ml of bone marrow were calculated. The yields were similar in N'Damas and Borans in the two age groups. Despite higher numbers of TNC in yearling cattle, the yields in BMMNC were similar to those of the adult cattle, indicating that the excess cells in TNC in yearling cattle are probably due to mature granulocytes. Adherent cells were removed from BMMNC by two successive, one-hour adherence steps on tissue culture flasks and the yields in non-adherent BMMNC (NA-BMMNC) per ml of bone marrow were calculated. The yields were similar in N'Damas and Borans in the two age groups. T-lymphocytes were depleted from the BMMNC using monoclonal antibodies to CD2, CD4 and CD8 and immunomagnetic beads (Dynabeads M450) and the yields in T-lymphocyte-depleted BMMNC (lymph-BMMNC) were calculated. The yields were similar in yearling N'Damas and Borans. Using *in vitro* clonogenic assays, each bone marrow cell fraction (BMMNC, NA-BMMNC and lymph-BMMNC) was assayed for its content in committed progenitors (CFU-E, BFU-E and CFU-GM). The yields in CFU-E and BFU-E per ml of bone marrow in the various cell fractions were similar in yearling N'Damas and yearling Borans and comparable between adult N'Damas and Borans. Adult cattle have slightly higher CFU-E (1.3 to 1.4 times) in BMMNC when compared to yearling cattle. Yearling cattle have 2.14 to 2.7 times more BFU-E in BMMNC than adult cattle. The yields in CFU-GM from BMMNC were similar in yearling and adult N'Damas, as well as in yearling and adult Borans but were consistently higher in Borans than in N'Damas. Altogether, these data reflected the homogeneous nature of the bone marrow aspirates collected from the cattle. Dilution of the samples with peripheral blood is unavoidable; however these data indicated that if dilution existed, its extent was similar in all the collected samples still allowing good comparisons of the animals involved. These data also showed the consistency in which the various cell manipulations and the clonogenic assays were performed in all individuals. The differences observed between breeds (CFU-GM from BMMNC) and between yearling and adult cattle (TNC, CFU-E, BFU-E) need to be confirmed in a large number of individuals before conclusive differences can be deduced. Clonogenic assays for CFU-E and BFU-E *in vitro* have been used to

evaluate the erythropoietic response of cattle following *Trypanosoma congolense* infection. Despite a nearly uniform preinfection status in cattle, considerable individual variation was observed following infection. This probably reflects the complexity of the disease and the multifactorial origin of the anaemia, each factor contributing to varying degrees in each individual animal.

### **T2d.2 Comparative effects of a primary infection with *Trypanosoma congolense* clone IL 13E3 on bone marrow erythroid progenitors from yearling N'Dama and Boran cattle**

**Scientists:** A. Andrianarivo, L. Logan-Henfrey, S. Mooloo, G. Gettinby\*

**Research Fellow:** H. Suliman

**Technicians:** P. Muiya, J. Ngatti, J. Kamau, B. Gichuki, J. Kabata

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Previous studies (*ILRAD 1993 Annual Scientific Report*, Abstract T2d.2) suggested that the trypanosome clone used for the infection can influence the response of the bone marrow to the anaemia. This study was undertaken to verify that assumption.

Four N'Dama and four Boran naive yearling cattle (12–16 months of age) were challenged with *T. congolense* clone IL 13E3 and the changes in the bone marrow levels of erythroid progenitors were evaluated over a 20-week infection period. Trypanosomes were detected between 14–16 days p.i. and reached a first peak between 21–27 days p.i. The overall parasitaemia levels were significantly lower in N'Damas than in Borans. The patterns of development of the anaemia were significantly different between the two breeds, with the mean packed cell volume (PCV) of the Borans decreasing at a much faster rate than those of the N'Damas. From week 9 p.i. onwards, the mean PCV gradually increased in the N'Damas and reached 30% by week 20 p.i. Over the same period, the mean PCV of the Borans levelled off at low values of 17.5–19%. The mean corpuscular volume (MCV) levels increased in both breeds, with those of the Borans increasing earlier and at a much faster rate, reaching maximum levels of 61 femtolitres (fl) in the Borans and 54 fl in the N'Damas at weeks 8–9 p.i. Thereafter they decreased and reached near preinfection values in the N'Damas by week 20 p.i., while they stayed around 47–48 fl in the Borans. The reticulocyte count (absolute percent) also increased in both breeds reaching maximum values of 2.3% at week 8 p.i. in the Borans, and 1.6% at week 8.5 p.i. in the N'Damas, dropping to preinfection levels thereafter. The bone marrow levels of CFU-E increased in both breeds reaching maximum levels of 380% of preinfection at week 5 p.i. in the Borans, and 320% at week 8 p.i. in the N'Damas. The bone marrow levels of BFU-E also increased in both breeds, with levels higher (but not statistically significant) in the Borans. The levels of both erythroid progenitors were decreased and were similar in the two breeds from week 10 p.i. onwards. This primary *T. congolense* IL 13E3 infection in yearling cattle demonstrates that in both breeds the bone marrow was initially responding to the developing anaemia and to a greater extent in the Borans. However, while the erythropoietic response resulted in partial

recovery of the mean PCV in the N'Damas, in the Borans the response did not appear to keep pace with the increased destruction of erythrocytes and no effect on the mean PCV was seen. The partial recovery of the mean PCV in the N'Damas may have resulted in diminished erythropoietin stimulation explaining the subsequent decrease in CFU-E and BFU-E levels, although they still remained above preinfection levels. In contrast, in the Borans, despite continued low levels of PCV from week 10 p.i. onwards, the numbers of CFU-E and BFU-E were also decreased, indicating insufficient erythropoiesis in the late stage of the disease.

The pattern of development of the anaemia described in this study agrees with that seen in previous *T. congolense* IL 1180 infections, but was different from that observed in the two previous *T. congolense* IL 13E3 infections indicating that the starting hypothesis was not verified. The reasons for this discrepancy are not yet known, though a close correlation was always noted between the erythrocyte indices (MCV, MCHC) in the peripheral blood and the erythroid clonogenic assays *in vitro* from the bone marrow. The yearling cattle in the present study controlled the disease better than the adult cattle in the previous *T. congolense* IL 1180 infection. The levels of parasitaemia were lower with intermittent periods of aparasitaemia in the N'Damas, the parasitaemia levels were lower in successive waves in the Borans, the erythropoietic response was faster and greater in the Borans. These data are in agreement with previous reports of resistance to trypanosomiasis in younger calves as opposed to adult cattle.

### **T2d.3 Enrichment of bovine haemopoietic progenitor cells by negative selection using monoclonal antibodies with magnetic beads**

**Technician:** P. Muiya

**Scientists:** J. Naessens, L. Logan-Henfrey, A. Andrianarivo

Haemopoietic progenitor cells in bone marrow can be enriched for their subsequent culture in clonogenic assays using monoclonal antibodies (MAbs) to cell surface antigens. Purification can be made by positive selection using MAbs to antigens on progenitor cells or by negative selection with MAbs that recognize surface antigens on non-colony-forming bone marrow cells. MAbs were generated against bovine bone marrow cells and others selected from the Second Ruminants Antibody Workshop. The cellular distribution of antigens on bovine bone marrow cells has been characterized using MAbs with flow cytometry and clonal assay cultures.

Bovine haemopoietic progenitor cells, like most of their progeny, expressed CD44 (IL-A118) MHC class I and WC9 (IL-A163) antigens. However, they did not express CD45 antigen, suggesting that MAbs to this antigen can be used to remove mature non-colony-forming progeny. CD11 $\alpha$  (IL-A99) was present on myeloid cells but not on erythroid progenitors, indicating that MAb IL-A99 can be used to enrich erythroid progenitor cells by negative selection. The same pattern of antigenic expression was observed for MAb IL-A55 which detects BoWC5 antigen. Progenitor cells did not express mature T lymphocyte antigens CD2 (IL-A43), CD4 (IL-A12) and CD8 (IL-A51) antigens. These antigens were how-

ever found on most bone marrow lymphocytes, suggesting that MAbs to the antigens can be used to enrich colony-forming cells by negative selection. A panel of MAbs was thus selected and used to enrich for haemopoietic progenitor cells by negative selection using magnetic beads.

MAbs Bo42 and GC6A (both CD45R) gave a 12- and 14-fold enrichment (respectively) of erythroid and myeloid progenitor cells. MAb IL-A55 (BoWC5) gave a 9-fold enrichment of both myeloid and erythroid progenitor cells. Burst forming unit-erythroid (BFU-E) were enriched 20-fold using the CD11 $\alpha$  MAb (IL-A99) but colony forming unit-erythroid (CFU-E) were enriched only 10-fold. This MAb however depleted most colony forming unit-granulocyte/macrophage (CFU-GM) colonies. A mixture of MAbs IL-A43(CD2), IL-A12(CD4) and IL-A51(CD8) gave a 10-fold enrichment. A cocktail of CD2, CD4, CD8 and CD45R MAbs attained a 20-fold enrichment of both erythroid and myeloid progenitor cells. Another cocktail mixture of CD2, CD4, CD8, CD45R and CD11 $\alpha$  MAbs enriched BFU-E colonies by 25-fold. This mixture depleted almost all CFU-GM.

Such a technique can be used to enrich haemopoietic progenitor cells in bone marrow for culture in short- and long-term cultures. It can also be used to selectively remove populations of bone marrow cells such as lymphocytes or monocytes/macrophages in studies to evaluate positive or negative regulation of erythropoiesis.

#### **T2d.4 Effects of removing bone marrow adherent cells and T-lymphocytes on erythropoiesis in cattle following a primary infection with *Trypanosoma congolense***

**Scientists:** A. Andrianarivo, L. Logan-Henfrey

**Technician:** P. Muiya

The erythropoietic response of cattle to the anaemia induced by trypanosome infection had been evaluated using unseparated bone marrow mononuclear cells (BMMNC). Beside the blood progenitor cells, this bone marrow cell fraction contains stromal cells (fibroblasts, endothelial cells, adipocytes) and accessory cells (macrophages, lymphocytes), which are responsible for the synthesis of stimulatory and inhibitory cytokines, the balance of which results in steady state haemopoiesis. During trypanosome infections, disturbances in accessory cell populations are known to occur. These disturbances may disrupt the cytokine balance and thereby the proliferation and/or differentiation of the progenitor cells. In clonogenic assays using unseparated BMMNC *in vitro*, the plated stromal cells and accessory cells are likely to secrete cytokines locally in the culture medium. The extent and the composition of these secretions may vary during trypanosome infection. Moreover, expansion of the accessory cell populations may result in dilution of the progenitor cells in BMMNC, and the numbers of colonies scored when assaying unseparated BMMNC may not reflect the full expression of proliferation and differentiation of the progenitor cells.

The present study was undertaken to explore the ultimate effects of bone marrow adherent cells (stromal cells and macrophages) and T-lymphocytes on the erythropoietic response in cattle following a primary *T. congolense* infection. For this

purpose, the progenitor (CFU-E and BFU-E) content in BMMNC was evaluated before and after removal of adherent cells (by 2 hour adherence on plastic flasks; NA-BMMNC) or T-lymphocytes (by negative selection using monoclonal antibodies to CD2, CD4 and CD8 immunomagnetic beads; lymph-BMMNC). Prior to the infection, removal of adherent cells or T-lymphocytes resulted in reduced growth of CFU-E and BFU-E (expressed as colony numbers per ml of bone marrow) in both N'Dama and Boran breeds of cattle. Consequently, the erythroid colony numbers were presented as a percent of preinfection for clearer presentation of the data. Following the infection, inconsistent increase or decrease in erythroid progenitor growth were noted in the Borans in NA-BMMNC or lymph-BMMNC, compared with BMMNC. In the N'Damas, BFU-E growth was consistently greater in NA-BMMNC until week 5 p.i., with maximum growth at 430% of preinfection and 340% of preinfection in BMMNC at week 5 p.i.; BFU-E growth was similar in BMMNC and NA-BMMNC from week 12 onwards. In contrast, BFU-E growth was consistently lower in lymph-BMMNC at all time points examined, except at week 7 and week 12 p.i., with maximum growth only at 240% of preinfection at week 8 p.i. However, it is not clear from the present data if the enhanced BFU-E growth *in vitro* was really due to the removal of adherent cells or to the presence of T-lymphocytes in NA-BMMNC. It is not known also if the inhibition of BFU-E growth was due to the removal of T-lymphocytes or the presence of adherent cells in lymph-BMMNC. The culture of non-adherent T-lymphocyte-depleted cells will provide an answer to those questions.

### **T2d.5 Macrophage structure and function in the bone marrow of *Trypanosoma congolense*-infected Boran cattle**

**Scientists:** V. Anosa\*, L. Logan-Henfrey

**Research Associate:** C. Wells

\*Department of Veterinary Pathology  
University of Ibadan, Nigeria

Smears and sections of sternal bone marrow (BM) collected by weekly sequential biopsy from five adult Boran cattle re-challenged with *Trypanosoma congolense* IL 13E3 were used to study the mononuclear phagocytic system (MPS) by light and transmission electron microscopy (TEM). Cells of the MPS including monoblasts, promonocytes, monocytes and macrophages increased several fold in both the bone marrow sinusoids and haemopoietic compartment during infection. The size of the bone marrow macrophages were significantly larger during the prepatent period compared to preinfection values. This suggested that macrophage activation was taking place in the bone marrow during the prepatent period. Morphologic features of macrophage activation included significant increases ( $P < 0.001$ ) in macrophage size and numbers of organelles including mitochondria, lysosomes and rough endoplasmic reticulum.

Macrophages phagocytized many non-mitotic haemopoietic cells (i.e. late precursors) of the erythroid and granulocytic series as well as mature erythrocytes, granu-

locytes and platelets but seldom lymphocytes. This was noted from day 29 post infection (days p.i.), when the first peak of parasitaemia occurred, until the termination of the experiment, 98 days p.i. Of 1200 macrophages examined by light microscopy, 25.8% had phagocytized recognizable cells including erythrocytes (18.6% of the macrophages), reticulocytes (4.5%), normoblasts (3.2%), neutrophils (3.3%), platelets (2.0%), eosinophils (0.5%) and lymphocytes (0.2%). Furthermore, 10.4% of these macrophages had phagocytized cells from more than one cell lineage. By TEM it was demonstrated that the process of cytophagia begins with cell to macrophage attraction, characterized by development of small filopodia of the cell membrane by the cell being engulfed to form contact points with the macrophages and of enveloping pseudopodia by the macrophage. This was followed by cell to macrophage adhesion along the entire contact surface and finally phagocytosis. The cells being phagocytized and those within vacuoles appeared morphologically normal and did not exhibit characteristics suggestive of apoptosis. Many macrophages were heavily-laden with haemosiderin during the chronic stage of the infection (78 and 98 days p.i.).

By TEM it was noted that during preinfection the small macrophages had contact with few haemopoietic cells. However, during the infection, the macrophages became enlarged and developed extensive pseudopodia that maintained contact with many haemopoietic cells through blunt reciprocal filopodia. Macrophages were not seen in the sinusoids of BM prior to infection but were numerous during infection, and adhered to sinusoidal endothelial cells by reciprocal blunt filopodia at the points of contact. The sinusoidal macrophages phagocytized blood cells (erythrocytes, neutrophils, platelets). Free trypanosomes though present in the arterioles of the BM, were never seen in the sinusoids or the haemopoietic compartment of the BM.

These studies demonstrate that the functions of the macrophages during trypanosomiasis include erythro- and leuco- phagocytosis which contribute significantly to ineffective haemopoiesis, increased iron storage and possibly impaired release of iron to erythroid precursors. The phagocytosis of cells from multiple lineages by an individual macrophage strongly suggests that a common mechanism of cell phagocytosis exists. Furthermore, circulating monocytes in blood are seldom noted to be cytophagic, and therefore these studies reemphasize the importance of the adhesion of macrophage to endothelial cells within the bone marrow sinusoids and in other organs in trypanosome-infected cattle. The presence of these resident, intravascular, activated macrophages within blood vessels suggests that they are the key cells responsible for haemolytic anaemia. A clearer understanding of what inflammatory mediators and adhesion molecules initiate this pathological reaction would shed light on why haemolytic anaemia due to extravascular haemolysis develops in trypanosome-infected cattle.

Macrophages thus appear to be key to both the development of ineffective haemopoiesis and extravascular haemolysis. The fact that this was a rechallenge infection prevents the determination of whether the early signs of macrophage activation during the prepatent period were due to immunological memory or were initiated by circulating inflammatory mediators in the absence of detectable trypanosomes.

**T2d.6 A morphological comparison of the haemopoietic response of adult N'Dama and Boran cattle during a primary *Trypanosoma congolense* IL 1180 infection****Scientists:** L. Logan-Henfrey, A. Andrianarivo, V. Anosa\*, S. Moloo**Research Associate:** C. Wells**Technicians:** P. Muiya, J. Kamau, M. Opollo, C. Ogomo, J. Kabata\*Department of Veterinary Pathology  
University of Ibadan, Nigeria

A detailed examination of the bone marrow response of cattle during a *T. congolense* IL 1180 infection was made in order to identify critical time points during the induction anaemia, the cell lineages affected and the factors responsible for the divergent course of the anaemia between N'Dama and Boran cattle. Three adult Boran cattle and three age-matched N'Dama cattle were exposed to a primary tsetse fly challenge with *T. congolense* IL 1180. Blood and bone marrow were collected weekly for haematology, clonogenic assays and RNA extraction (*ILRAD 1993 Annual Scientific Report*, Abstract T2d.2 and T2d.8), cytology, histology and transmission electron microscopy (TEM) over 17 weeks (119 days).

The total number of nucleated marrow cells (TNC) collected for clonogenic assays were consistent with the grossly observed cellularity of the BM samples collected for cytology. The mean number of TNC during preinfection were similar in the two breeds. The number of TNC dropped by day 7 post infection (days p.i.) to 89% in the N'Dama and 66% of preinfection levels in the Boran cattle. While the numbers of cells remained below preinfection in the Boran cattle throughout the study, a nonsignificant peak at 140% of preinfection occurred in the N'Dama cattle on 49 and 63 days p.i. During the chronic phase of disease (98–119) the number of TNC in the N'Dama were below preinfection but were higher than in the Boran. Nonetheless, there were no significant breed differences, nor a significant breed  $\times$  time interaction throughout the experimental period. In support of the TNC counts it was recorded on 49 days p.i. and thereafter that the bone marrow from N'Dama cattle was markedly more cellular than that of the Boran cattle. Between 70–119 days it was difficult to collect liquid bone marrow from the Boran cattle often requiring repeated biopsy before successful bone marrow aspiration. Concurrently, bone marrow aspirates from the N'Dama cattle were easily collected and remained highly cellular. It is suspected that the method of counting TNC may result in the lysis of late erythroid normoblasts as well as the non-nucleated reticulocytes and mature erythrocytes. Thus calculated numbers of TNC would not take into account these late cells in the erythroid series.

As anaemia developed in both breeds of cattle during the acute infection, there was a shift within the bone marrow in the ratio of granulocytic to erythrocytic precursors towards the erythroid lineages as noted by cytology, histology, TEM and clonogenic assays. The intermittent granulocytopenia which developed in the blood of Boran cattle can be explained by the depression in immature granulocytic progenitors as detected by CFU-GM clonal assay and depression in granulocyte precursors

as noted by cytology. In contrast, a significant peak above preinfection of CFU-GM was observed in the N'Dama cattle on 49 days p.i. which may explain their maintenance of the numbers of granulocytes and monocytes in the blood at levels that fluctuated close to preinfection levels.

The number of macrophages was increased in both groups of cattle throughout infection. The mean size of macrophages in preinfection was significantly larger in Boran than in N'Dama cattle. From 15 days p.i., coincident with the first peak of parasitaemia, the size of macrophages in both groups of cattle increased. The macrophages of the N'Dama became significantly larger than those of the Boran cattle. From 28 days p.i. onward the N'Dama cattle had a higher percentage of cytophagic macrophages containing more phagocytosed cells than the Boran cattle. Thus it would appear that bone marrow macrophages of N'Dama are more highly activated as judged by increase in size and their higher phagocytic index.

In a retrospective examination of previous studies the percentage of macrophages and phagocytic index was compared in *T. vivax*, *T. congolense* IL 13E3 and *T. congolense* IL 1180 infections with clinical outcome of disease. It was found that the *T. vivax* infection resulted in the highest proliferation of macrophages followed by *T. congolense* IL-13E3 and finally *T. congolense* IL 1180. Individual animals with greater cytophagia by bone marrow macrophages developed a better haemopoietic response and less severe anaemia.

It should be remembered, however, that hyperplasia of the mononuclear phagocytic system (MPS) during trypanosome infections is known to occur in other organs such as the spleen, liver, lungs, adrenals, pituitary, lymph nodes and haemolymph nodes. The overall balance of erythrocyte removal by these resident macrophage populations can best be assessed using erythrokinetic studies with some supportive histology and TEM. Previously, it has been reported that the half-life of erythrocytes in blood was shorter in *T. congolense*-infected zebu than in N'Dama cattle thus demonstrating that the overall extravascular haemolysis is higher during the acute phase of disease in zebu cattle.

During the chronic phase of the anaemia, marked poikilocytosis was noted in the blood of the remaining two Boran cattle and slight poikilocytosis in one of the three N'Damas. Poikilocytes are reported to be selectively removed by macrophages and, therefore, an animal that has many such cells would be expected to have erythrocytes with a greatly shortened half-life.

The N'Dama cattle developed mild anaemia in contrast to the severe anaemia seen in the Boran cattle and therefore would not necessarily be expected to have a higher bone marrow response than Boran cattle as assessed by clonogenic assays *in vitro*. Initially, it would seem a paradox that there was a higher rate of cytophagia by macrophages in the N'Dama cattle compared with Boran cattle. Macrophages are known to play both an important positive and negative role in haemopoiesis and it is the balance of these roles that is critical to maintaining steady-state haemopoiesis. The roles of erythroid potentiating factor and erythropoietin may be critical to the maintenance of this balance and are being investigated.

**T2d.7 Cloning of bovine glyceraldehyde-3-phosphate dehydrogenase cDNA****Scientist:** B. Mertens**Research Associate:** L. Gaidulis**Technicians:** C. Muriuki, Y. Verjee

Analysis of gene expression using RT-PCR is a valuable technique for determining the presence or absence of a particular mRNA. An endogenous sequence, known to be present at constant levels throughout a series of samples to be compared, can be used as an internal standard in quantitative RT-PCR reactions. Unfortunately, few genes are expressed in a strictly constitutive manner. This is even the case for many housekeeping genes, including beta-actin. It has been shown that  $\beta$ -actin levels increase three- to six-fold in bovine lymphocytes activated with concanavalin A for 10 or 24 hours respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has proven to be a better internal control than beta-actin and is now widely accepted for this purpose.

The sequence for bovine GAPDH gene has not been reported previously. The bovine analogue was cloned by PCR technology, using primers based on the human GAPDH sequence. A 935 bp fragment was sequenced which shows 90% homology at the nucleotide level with human GAPDH.

Based on this sequence, a bovine specific primer set was designed to be used in further RT-PCR assays. Experiments are currently under way to compare GAPDH and  $\beta$ -actin mRNA expression in mitogen stimulated bovine cells in a quantitative PCR study (see Abstract T2c.3).

**T2d.8 Cloning of bovine interleukin3 (IL-3) cDNA****Research Fellow:** S. Mwangi**Scientists:** B. Mertens, L. LoganHenfrey

The cloning of a 533 bp bovine IL-3 cDNA fragment has been previously reported with the aim of elucidating the role of the cytokine in bovine haemopoietic stem cell differentiation (see *ILRAD 1993 Annual Scientific Report*). Based on this sequence, a bovine-specific 5' primer was designed and used to amplify an additional 363 nt from the 3' non-coding region according to the RACE (random amplification of cDNA ends) protocol. In this region and upstream of the polyadenylation site are six 'ATTTA' repeat units as reported in the human IL-3 cDNA sequence. These sequence motifs have been shown to mediate selective messenger RNA degradation. Comparison of the IL-3 cDNA sequence, both in the translated and untranslated regions, amplified from Boran and N'Dama cattle revealed no sequence differences.

The 144 amino acids (aa) encoded by the bovine IL-3 cDNA include a 17 aa putative signal peptide whose cleavage leaves the mature protein of 14.5 kDa. The mature protein lacks the cysteine residues required for disulphide bridge formation found in the human and murine proteins. Cysteine residues are also reported to be missing in the ovine protein. Computer-assisted secondary structure predictions

have, however, shown that the protein will fold into four helices similar to the human and ovine proteins.

Studies with the human and murine proteins have demonstrated that the level of glycosylation does not influence the biological activity of the protein. Therefore, to obtain recombinant bovine IL-3, the cDNA sequence coding for the mature protein has been cloned into the Pinpoint Xa-1 expression vector (Promega). In this system the protein is expressed in *Escherichia coli* as a fusion protein with a naturally biotinylated tag which can be purified utilizing the interaction of biotin with avidin. A protein of the expected size ( $\approx 28$  kDa) has been detected in total *E. coli* cell lysates, and the protein purification conditions are being optimized.

Total RNA samples have been isolated from peripheral blood lymphocytes (PBLs) collected from normal and *Trypanosoma congolense*-infected Boran and N'Dama cattle and are being used to investigate IL-3 gene expression by semi-quantitative polymerase chain reaction (PCR).

## T2d.9 Molecular cloning of the transcript encoding bovine erythropoietin

**Research Fellow:** H. Suliman

**Scientists:** L. Logan-Henfrey, B. Feldman\*, B. Mertens, P. Majiwa

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The major pathophysiological change associated with trypanosomiasis in livestock is anaemia. An inadequate erythropoietin (EPO) response has been suggested as one of the possible mechanisms that may contribute to the non-responsive anaemia seen during trypanosome infections, which is particularly evident during the chronic stage of the disease. EPO is a glycoprotein which is unique among the haemopoietic growth factors in functioning as a blood stream hormone with the bone marrow as its target organ. It is essential for proliferation and terminal differentiation of erythroid progenitors in the bone marrow. In adult animals it is produced in the kidneys; the liver can express EPO mRNA in foetal life and during severe anaemia.

A bovine kidney cDNA library constructed in Uni-ZAP XR was screened with a bovine EPO cDNA fragment (723 bp) amplified in a polymerase chain reaction (PCR) using oligonucleotide primers based on the published sequence of sheep EPO. Of the positive plaques identified, only two were found to have inserts of sufficient length; 1214 bp and 1243 bp. These two inserts have the poly-A tail but only 43–49 bp of the 5' untranslated region. We have used the 5'RACE (random amplification of cDNA ends) technique to amplify the most extreme 5' untranslated region of bovine EPO. We have determined that the full sequence of bovine EPO cDNA is 1386 bp in length and has an open reading frame of 579 bp, encoding 168 amino acids (aa) of mature protein with a calculated molecular weight of 18.4 kDa and presumptive signal peptide of 24 aa. Northern blot analysis revealed that EPO mRNA exists as a transcript of approximately 1.4 kb. The Northern blot analysis can detect EPO mRNA

only in kidneys from anaemic cattle and from the liver of a bovine foetus. The bovine EPO cDNA exhibits 94%, and 88.5% homology at the nucleotide level to sheep and human EPO cDNA, respectively. When compared at the aa level the bovine EPO has 92% and 82% homology to that of sheep and human respectively.

The bovine EPO cDNA has been subcloned into a pcDNAINEO vector in readiness for transient expression in COS cells. Stable expression of this hormone will be carried out in CHO cells.

### **T2d.10 Determination of the relative quantities of EPO mRNA in kidneys and livers from cattle chronically infected with *Trypanosoma congolense***

**Research Fellow:** H. Suliman

**Scientists:** L. Logan-Henfrey, B. Feldman\*, B. Mertens, P. Majiwa

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Virginia Tech, Blacksburg, Virginia, USA

Blunted erythropoietin (EPO) production has been suggested as one of the possible mechanisms that contribute to the non-responsive anaemia associated with trypanosome infections in livestock. Haemolytic and/or haemorrhagic anaemia in human and sheep are known to elicit substantial increases in plasma EPO, accompanied by marked increases in the transcription of EPO mRNA in the kidneys and liver. It has been observed that the level of EPO production is inversely proportional to the haematocrit level. The transcription of EPO mRNA in kidneys and livers has been investigated during chronic *T. congolense* infection in five N'Dama and five Boran cattle, in an uninfected steer, in a bovine foetus, and in a calf made severely anaemic by phlebotomy.

Total RNA was prepared from kidneys and livers collected from the chronically infected Boran and N'Dama cattle. Relative quantities of EPO mRNA in each tissue was determined using Northern blot hybridization and semiquantitative reverse transcription and polymerase chain reaction (RT-PCR). Using Northern blot analysis, EPO mRNA was not detected in livers or kidneys of the non-infected steer or in livers of infected cattle, but was detectable in liver and kidney from the severely anaemic calf, bovine foetal liver and kidneys of the infected cattle. Preliminary results using RT-PCR technique, revealed approximately a six-fold increase in the EPO mRNA in the kidneys of the trypanosome-infected cattle (Boran and N'Dama) compared to the level of EPO mRNA in the non-anaemic steer. EPO mRNA in the kidneys of the piebotomized calf was five-fold greater than those in the kidneys of the trypanosome-infected cattle. The five N'Dama cattle infected with *T. congolense* were found to have similar average increases in EPO mRNA compared to the average increases in the five Boran cattle infected with the same parasite. Surprisingly, the five Boran cattle were more anaemic, with haematocrit levels ranging between 15 and 19%, while the five N'Dama cattle had haematocrit values between 25 and 33%. This suggests that EPO mRNA might indeed be reduced in Boran cattle chronically infected with *T. congolense*.

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# RUMINANT GENETICS

### **G1a.1 Development and phenotyping of a bovine F2 trypanotolerance resource population**

**Scientists:** A. Teale, D. Kennedy, S. Moloo, S. Kemp

**Technologists:** J. Ngatti, H. Gathuo, M. Ogugo, J. Kabata, S. Leak, R. King

A major experiment is being conducted to mark and then identify the genes of N'Dama cattle responsible for their relative tolerance of trypanosomal infection. A resource population of crossbred animals between trypanotolerant N'Dama cattle and susceptible Boran cattle is being developed. During 1994 two full-sibling families of N'Dama × Boran F2 cattle have been completed with 39 and 32 calves born, and nine confirmed pregnancies in surrogate dams. Two further F2 families have been brought to an advanced stage, with 16 and 12 calves born and five confirmed pregnancies. A number of other families were initiated giving an overall total of 116 F2s born and 39 *in utero* at the end of August 1994. Forty-one embryos in two families have been cryopreserved in liquid nitrogen.

F2 animals have been reared on their dams at Kapiti Plains Estates near Nairobi, in a tsetse fly-free area, until weaning at eight months of age. After a further two months, animals have been moved to the Laboratory for a two-month period of acclimatization prior to trypanosomiasis challenge at 12 months of age.

Phenotyping of the first group of animals for trypanotolerance was initiated in December 1993 and then continued as successive groups of F2 animals reached the challenge age. In all cases, monitoring was initiated three weeks prior to challenge, with peripheral blood parasite counts, PCV, total and differential peripheral blood leukocyte counts, rectal temperature and body weights being recorded on a regular basis. Monitoring was continued for 150 days after infection. Plasma and serum samples were taken at each sampling for cryopreservation in liquid nitrogen. Challenge was with *Trypanosoma congolense* clone IL1180 delivered through the bites of eight tsetse flies (*Glossina morsitans centralis*). By September 1994, 14 animals had completed the 150-day monitoring period after challenge, with another 27 being monitored following challenge. At the end of 1994, it is anticipated that 27 animals will have completed the phenotyping exercise and another 38 will be at various stages in the challenge process.

Considerable variation in response has been observed in the 14 animals which have completed phenotyping. At one extreme, one animal suffered a modest fall in PCV to 62% of preinfection values on day 60 after challenge, before recovering to a PCV of 96% of preinfection levels at day 150. Over the course of the challenge this animal continued to gain body weight. At the other extreme, one animal suffered a fall in PCV to 34% of preinfection levels on day 95 after infection, and on day 150 had a PCV of 39% of preinfection levels. This animal lost 14% of its body weight over the course of the monitoring period. There was also considerable variation in the levels of parasitaemia observed, especially of the first peak. Generally there was close correlation between levels of parasitaemia and clinical illness, judged on the basis of PCV, changes in circulating leukocyte levels and body weight changes.

### **G1a.2 Characterization and linkage mapping of a panel of polymorphic bovine microsatellites**

**Scientists:** S. Kemp, O. Hishida, A. Teale, J. Hetzel\*, A. Crawford†, G. Guerin‡

**Technologists:** J. Wambugu, M. Ogugo

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Work at ILRAD is contributing to the international collaborative effort to map the bovine genome. At the end of 1993 a panel of approximately 100 polymorphic bovine microsatellite markers had been developed at ILRAD. At that point production of microsatellites ceased and their mapping and characterization begun.

The polymorphism of all of these bovine microsatellites has now been characterized with reference to a small population of diverse breeds and their polymorphism information contents (PICs) have been calculated. The mean PIC is 0.71, confirming the expected high level of informativeness.

The performance of all markers in goat and sheep has also been determined—33% are informative in goat and 40% in sheep. A selection of markers informative in sheep will be incorporated into the sheep map by the group at Dunedin.

Physical assignments to chromosomes have been made for 15 markers in collaboration with INRA. Physical assignment is no longer receiving a high priority as it generally yields less useful information than linkage mapping.

Linkage mapping of the markers is under way; 22 have been incorporated into the linkage map in collaboration with CSIRO and a further 20 have been typed against the approximately 300 animals which comprise the international bovine reference panel. This information will shortly be used to linkage map these markers, and it is hoped to complete the linkage mapping before the end of 1994.

Arrangements have been made with GenBank for a large-scale submission of the marker sequences together with associated information. Approximately 100 mapped and characterized ILRAD microsatellite markers are therefore expected to be in the public domain before 1995.

### **G1a.3 Polymorphic genetic markers in specific bovine genes**

**Research Fellow:** M. Agaba

**Supervisors:** S. Kemp, A. Teale, B. Perry

DNA sequence polymorphism in four genes identified by direct DNA sequencing has been reported previously, as well as their subsequent assay in populations using allele specific amplification (ASA) or polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques. In continued attempts to identify polymorphism in specific genes of interest, single strand conformation polymorphism (SSCP) analysis has become the method of choice.

DNA sequence variation has been detected and assignments made to syntenic groups for the 17 genes listed. Unless otherwise stated, assignments were made through work at ILRAD: The gene for  $\beta$ -arrestin has been assigned to syntenic group U19, vimentin to U11 (from published data), T cell receptor  $\beta$  to U13 (from published data), glutathione peroxidase 1 to U12, glutathione-S-transferase to U19, alpha-1-3-galactosyltransferase to U16, galactosyltransferase is unassigned, pituitary adenylyl cyclase activating peptide to U28, regulatory anchor protein is unassigned, membrane calcium pumping protein is unassigned, ribonucleoprotein-SSB to U17, adenylylate kinase isoenzyme 2 to U17, desmocollin to U28, ornithine decarboxylase to U16, superoxide dismutase 2 to U2 (from published data), cartilage proteoglycan is unassigned, lens aldose reductase pseudogene is unassigned.

Genotypes of informative families in the international bovine reference family panel have been determined and linkage analysis of 10 genes has allowed their incorporation into the bovine linkage map. Genotyping and linkage analysis of the remaining genes is expected to be completed in the next six months.

The genes for erythropoietin, interleukin 3, coagulation factor V, N-acetylglucosamine, ( $\beta$ -1-4) galactosyl transferase and plasma glutathione peroxidase, each expected to map to a different chromosome, will be investigated.

In addition, a more detailed study will be undertaken to determine the cattle equivalent(s) of human chromosome 18 (HSA18). This will allow comparison of the organization of this region in the bovine, human and murine genomes. The approach will be to identify polymorphism in bovine homologues of selected HSA18 genes (N-cadherin, alpha laminin, transthyretin, ferrochelatase, thymidylate synthase, myelin basic protein and gastrin releasing peptide). Somatic cell genetics has been used to assign four HSA18 genes (desmocollin, N-cadherin, cytochrome B5 and PACAP) to cattle chromosome 24. This provides the first indication that a large part of cattle chromosome 24 is equivalent in terms of gene content to HSA18.

#### **G1a.4 Mapping of markers associated with survival time of inbred mouse strains challenged with *Trypanosoma congolense***

**Scientists:** S. Kemp, A. Teale

**Technologists:** H. Gathuo, J. Mwakaya, M. Ogugo

In order to identify markers of genes associated with survival time of inbred mouse strains challenged with *Trypanosoma congolense*, Balb/c  $\times$  C57/bl6 F2 and back-cross populations were established and challenged with *T. congolense* (IL1180) in December 1992. The mean time to death of the parental stocks was 68 days for the Balb/c and 142 days for the C57/bl6. The F2 (399 mice) showed a mean time to death of 91 days and the backcross to the Balb/c parent (376 mice) had a mean survival time of 82 days. The F2 and back-cross mice showed a range of survival times which included those of both parental populations.

Subsequently, a selective genotyping of F2 mice has been carried out in which 40 mice, 20 of those with the shortest survival time and 20 of those with the longest survival time, have been genotyped with 87 polymorphic microsatellites. Markers

which showed preliminary evidence of association with survival time are being used to type more mice with extreme survival times, such that a total of about 30% of the challenged mice will be genotyped. The initial genotyping data has been analysed by the MAPMAKER programme and this has proved useful in detecting errors in published gene orders and in our genotyping. It has shown that about 85% of the mouse genome has been included in this initial screen and another 15 markers to cover the remaining 15% will now be applied. Once this has been achieved, analysis with MAPMAKER/QTL will be performed to identify potential quantitative trait loci (QTL) influencing survival in these mice. Initial examination of the data already indicates one area of the genome with a large effect.

In April 1994 a challenge of a second mouse population based on a cross between the susceptible A/J strain and the C57/bl6 was performed. Eight hundred and sixty F2 mice, 55 mice of each parental line and 55 F1 mice were challenged with IL1180. This challenge is nearing completion and there are clear differences between the groups. The times to 50% death within each group are as follows: A/J 52 days, F2 101 days, C57/bl6 110 days, F1 137 days. To date all of the 100 unchallenged controls survive. There was a spread in times to death in the parental lines, but when 90% of the A/J mice had died only 6% of the C57/bl6 were dead. F2 mice cover this full range of susceptibility and F1 mice were more resistant than either parent. Genetic analysis of the F2 population will begin when all mice have either died or become aparasitaemic. The larger number of mice in this F2 generation will allow a higher resolution QTL mapping than in the previous Balb/c  $\times$  C57/bl6 cross. This challenge will also provide an indication of whether or not the same QTL are playing the same roles in these different crosses. In order to further increase the mapping resolution, small randomly bred intermediate generations will be used to generate a large F5 or F6 which will be challenged. The extreme phenotypes will be genotyped with markers of QTL identified in the F2 generation. The additional recombination events between the F2 and the F5 or F6 will then allow for very high resolution mapping of genes controlling survival of *T. congolense*-infected mice.

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# SOCIOECONOMICS

**EP1.1 Quantitative assessment of the relationship between infections with vector-borne pathogens and production loss under different livestock production systems**

**Scientists:** B. Perry, R. Kruska, P. Lessard, S. Morzaria, A. Mukhebi, A. Young, M. Burrige\*, S. Mahan\*, J. McDermott†, G. Medley‡, R. Norval§, W. Thorpe¶, U. Ushewokunze-Obatolu\*\*

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The objective of this activity is to determine the effects of infections by vector-borne pathogens on ruminant livestock productivity, in order to be able to predict the economic impact of these infections under different conditions, and the relative merits of different control options. The activity has two components. The first is the development of broad-based models of tick-borne infections which will help to explain their epidemiology and predict their effects on livestock production, with particular reference to sub-Saharan Africa. This includes the development of georeferenced digital databases on livestock production systems in the region, to permit a better characterization of current and developing production systems in terms of their susceptibility to the effects of tick-borne infections. The second component is a series of case-studies in different countries and production systems to generate data for the broad-based models, and eventually to test them out.

A case-study of the impact of tick-borne infections on smallholder dairy production in Murang'a District, Kenya, considered typical of many smallholder dairy systems in eastern Africa, has been initiated. The project aims to quantify the relationships between the incidence of tick-borne infections and productivity loss, and in so doing evaluate the role of antibody prevalence data as an indicator of endemic stability and instability. In the first phase of this project, a cross-sectional study of the prevalence of tick-borne infections by agroecological zone was carried out, and the results were related to the main characteristics of dairy production systems in the District. Antibody prevalence rates to *Theileria parva* varied by agroecological zone (from 18% in the lower highlands to 72% in the upper midlands), by breed (from 16–28% in improved breeds to 86% in zebu breeds) and by grazing management (from 30% in zero grazing to 63% in open grazing systems). Three contrasting areas of the district have now been selected on the basis of these results to assess the relationship between apparent endemic stability and instability, as measured by antibody prevalence estimates, and productivity losses.

In early 1995, the laboratory will initiate a collaborative project with the USAID-funded Heartwater Research Project in Zimbabwe, being carried out as a subcontract from the University of Florida. Under this collaboration, the results of efforts to develop transmission dynamics models of theileriosis, work being undertaken in collaboration with the University of Warwick, will be extended to heartwater.

A further case study, the objective of which is to evaluate the productivity effects of persistent infections with *Cowdria ruminantium* (the cause of heartwater) is being initiated in Zimbabwe. In this study, the effects of artificially-induced infections in persistently infected small ruminants on reproductive performance, milk yield and growth rates are being studied under experimental conditions.

### **SEP1.2 The economic impact of vector-borne diseases and their control**

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The direct economic cost (or losses) caused by theileriosis and the economics of an alternative control strategy based on immunization by the infection-and-treatment method have been assessed at sites in several of the affected countries by applying a computer spreadsheet model; results have been reported previously. During 1994, the spreadsheet model was adapted and applied to a similar analysis of heartwater in Zimbabwe in collaboration with the University of Florida UF/USAID/SADCC Heartwater Research Project and the Veterinary Research Laboratory in Harare.

The economic cost of heartwater and the economics of its potential control by the infection-and-treatment method of immunization in Zimbabwe were assessed in both the communal lands and large-scale commercial farming systems under different scenarios of disease spread (no spread to country-wide spread) and control (effective control based on acaricides or immunization). Preliminary results show that the estimated annual national economic cost of heartwater in Zimbabwe in 1992 under the current control strategy based entirely on acaricide application was Z\$ 56.8 million (US\$ 8.7 million; discounted at the rate of 20%). Of the total economic cost, 45% was incurred in the communal lands and 55% in the large-scale commercial farms.

The annual discounted economic cost per animal under the current control strategy was calculated to be Z\$ 10 in the communal lands and Z\$ 34 in the commercial farms.

A control scenario assuming successful restriction of the disease to its current distribution in the lowveld using acaricide application would result in annual economic cost per animal of Z\$ 13 in the communal lands and Z\$ 21 in the commercial farms. Under a scenario assuming the gradual spread of the disease, with control through an immunization strategy, the economic cost per animal would be reduced to Z\$ 3 and Z\$ 10 in the communal lands and the commercial farms respectively. These results indicate that even if the current effective dipping practice would continue and the spread of heartwater successfully contained, the economic costs (largely acaricide costs) in both production systems would still be substantial and an immunization strategy would likely be the most cost-effective.

An immunization based control strategy would substantially reduce the total annual national economic cost of the disease to Z\$ 9.0 million (discounted at 20%), representing a 70% reduction in the annual national economic cost. Sensitivity analysis showed that immunization cost per animal (estimated at Z\$ 12 in this analysis) would have to rise to Z\$ 320 for the communal lands cattle, and Z\$ 1600 for the cattle on commercial farms, to reach the break-even levels. However, it is considered unlikely that routine immunization costs would approximate these break-even levels, implying that immunization is an economically viable proposition, and financially superior to the current control strategy based on the application of acaricides.

The computer spreadsheet model used for the assessments of the economic costs of tick-borne diseases and their control has been adapted for conducting similar case study analyses for trypanosomiasis. The aim of the case studies is to obtain data from different major livestock production systems and agro-ecological zones, which will be analysed to provide more reliable quantitative assessments of continent wide economic costs of trypanosomiasis. In addition, the information from individual case studies will help policymakers choose the most cost-effective and sustainable control measures for specific areas and circumstances.

In collaboration with A.P. Consultants in the UK, the spreadsheet model approach has been applied to estimate the economic costs of trypanosomiasis in selected countries (Cote d'Ivoire, The Gambia and Zimbabwe) and results have been reported previously. During 1994, work on an additional case study in Cameroon has continued, in collaboration with the University of Reading. The objectives of the research include: to assess tsetse and trypanosomiasis as a constraint to livestock production in Cameroon; to assess the economic impact of effective tsetse control by aerial spraying in Adamawa Province of Cameroon; to analyse the economics of alternative sustainable tsetse control methods and the willingness to pay for them by livestock keepers; and to examine the environmental consequences of tsetse and trypanosomiasis control in the Adamawa Province. The data are currently being analysed. The results of this study will be used to supplement the continental assessment of the impacts of trypanosomiasis and its control in Africa.

**SEP1.3 The impact of tsetse control on land-use change, vegetation structure and biodiversity**

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The objective of this research activity is to assess the impacts of controlling trypanosomiasis on land-use change and ecological properties. The research approach integrates a continental and national-scale geographical information systems (GIS) analysis with field case studies on eastern, western and southern Africa. In Ghibe Valley, Ethiopia, a case study was initiated by ILRAD and ILCA to quantify the impacts of disease control on livestock numbers and land use by comparing changes in livestock holdings with the area of land cultivated by farmers. First, a cross-sectional household survey was completed that compares recent changes in livestock holdings and land-use for households in an area where ILCA has controlled the fly with insecticidal pour-ons since January, 1991, and for households in areas where there has been no control of the tsetse fly. Second, for a sub-sample of these households, the total area of cultivated fields was measured on the ground and compared with field size estimates made by farmers during the household survey. Third, on a larger scale, a Landsat Thematic Mapper satellite image for 1993 was obtained and used to develop a land-use/land cover map of the valley as a first step in an historical study of land-use change.

Preliminary results from the household surveys and cultivated field measurements show that, as expected, there were significantly more cattle (particularly oxen) in the tsetse-free than the tsetse-infested areas. However, it was not known whether the farmers in the tsetse-free area held more cattle than farmers in the infested areas at the beginning of the control programme. More indicative of the effects of tsetse control were the decreased mortality rates in cattle and the faster rate of increase in field size in the tsetse-free compared with tsetse-infested areas. Thus, from 1991–1993, it appears that tsetse control began to spur expansion of agricultural land-use in Ghibe Valley.

The satellite image was classified using a supervised computer classification and the results were compared with 130 ground-truth points and transects collected using a GPS (Global Positioning System) device in the field. The computer-generated classification correctly distinguished cultivated fields from surrounding woodlands and grassland with an accuracy of 75%. The map was used to test the relationship between the distribution of tsetse and land-use by assuming that tsetse infestations

reached an elevation of about 1700 m. Land-use intensity decreased from 1700 to 1300 m elevation, possibly as a function of the increasing tsetse challenge. However, below 1300 m, where tsetse infestation was ubiquitous, land-use intensity was highest along the edge of rivers which often flowed through the lowest points in Ghibe Valley.

The second part of this research is to quantify the effects of land-use change initiated by tsetse control on ecological properties. A preliminary study of the effects of land-use change on vegetation structure and bird biodiversity in Ghibe Valley, Ethiopia were completed in 1994. In both tsetse-infested and tsetse-free sites, vegetation structure and biodiversity were compared across a gradient of four land-use/land cover types from low to high land-use intensity, including riparian woodlands (along streams and rivers), wooded grasslands, smallholder fields within grassland mosaics, and large-holder cultivation. In these four land-use types, woody plant cover and density, tree height and diameter, herbaceous plant cover and height were measured along with the number, composition and abundance of bird species in 70 plots.

It was expected that the abundance of vegetation, its structural complexity and the diversity of bird species would be highest in the riparian woodlands and would decrease across the land-use gradient as human use increased. Indeed, riparian woodlands represented unique habitats on the landscape; they were structurally complex and sheltered tree and bird species found nowhere else in Ghibe Valley. Wooded grasslands were much less species-rich than riparian woodlands, and were most often converted to smallholder, oxen-ploughed cultivation as a result of tsetse control. Unexpectedly, cultivation of these woodlands resulted in no significant change in vegetative abundance or bird biodiversity. The largest change in vegetative structure and biodiversity occurred when wooded grasslands were converted to large-holder farms, which were ploughed exclusively with tractors. Clearly, this conversion was not a result of tsetse control. These results imply that tsetse control, if it results in conversion of less-used lands to smallholder farms, will have minimal impacts on vegetation and bird biodiversity. However, changes in land-use caused by tsetse control may be expected to have marked impacts on vegetative structure and bird biodiversity if it results in the conversion of riparian woodlands to smallholder cultivation or woodlands/grasslands to large-holder cultivation.

The relationship between human use and large mammal biodiversity in Ghibe Valley, however, appears to be quite different than that for vegetation and birds. A farmer survey was conducted to determine the historical and current distribution of different species of large mammals. Results show that, before the tsetse control started, large mammal populations were decimated due to human use. This implies that tsetse control, if it encourages influx of human populations into other, largely unpopulated ecosystems, could have significant impacts on the biodiversity of large mammals.

Extension of the field investigations to the Zambezi Valley began in 1994 with development of a study design and a workshop with collaborators in Harare. Plans for 1995 include extension to a West African study site, beginning of research in the Zambezi Valley and continuation of research in the Ghibe Valley of Ethiopia. A continental GIS analysis of the relationship between tsetse fly distribution and land use, initiated in 1993, was continued, and the data acquisition phase was completed during 1994. Analysis is currently under way.

**SEP 2.1 Development, acquisition and dissemination  
of digital georeferenced databases**

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The objective of this activity is to develop and acquire digital databases on specific variables and at the appropriate resolution required for modelling, research and decision-support needs in the area of improved animal health. ILRAD has been designated a lead centre for geographical information systems (GIS) within the CGIAR, and the Socioeconomic and Environmental Impact Programme has played an active role in the CGIAR/UNEP initiative to identify and develop digital databases of common interest to the research needs of international agricultural research centres.

Current research at ILRAD on the epidemiological, socioeconomic and environmental impacts of controlling animal diseases, which make use of vector, disease and economics models and decision-support systems, requires extensive georeferenced databases that can be integrated in a GIS environment. The GIS is a powerful tool that allows the integration of these interdisciplinary research efforts. In the past year, a variety of climatological, environmental, demographic and land-use data layers at various resolutions of scale (local, national and continental) have either been developed within ILRAD, or acquired from collaborating institutions. Major collaboration between international agricultural research centres has been greatly enhanced by the CGIAR-GIS initiative begun in 1991 that has the responsibility of defining the common database needs of all the centres, and acquiring the necessary funding for further database development. Collaboration has also continued with other international centres, national agricultural research services and the United Nations Environment Programme.

At the local level extensive database development has occurred for the study site in the Ghibe Valley, Ethiopia, ( $\approx 1500$  sq km) for the purpose of analysing the potential land-use impacts resulting from tsetse control. To put the high resolution Ghibe Valley study site research results into a national context. Ethiopia-wide databases have also been acquired. ICRAF has recently developed interpolated

climate surfaces at a 1 km resolution for the country including long-term monthly mean minimum and maximum temperature as well as rainfall. These surfaces are based on a 1 km digital elevation model developed by the United States Geological Survey (USGS). USGS and the Famine Early Warning System (FEWS-USAID) provided land use, soils, human population, agricultural productivity and administrative boundary data layers.

Other national datasets acquired include a detailed 1994 land use layer for Zimbabwe, developed by FEWS, Harare. This layer, which is based on ward boundaries, includes other information such as human population numbers. Such information will be an important addition to the animal health decision-support system installed by ILRAD in the Zimbabwe Veterinary Research Laboratory earlier in the year, as well as ILRAD's continuing research to model various tick and disease distributions and dynamics in the region. This system currently consists of over 90 georeferenced data layers including veterinary infrastructure, country infrastructure, disease outbreaks, vector distributions, wildlife host distributions, natural resources, climatic and demographic themes. In addition, databases for the northwestern region of Zimbabwe were acquired from the Natural Resources Institute (NRI) in the UK. These include land use, human population distribution, geomorphology and vegetation types. These data will be used in the case study of the environmental impacts of tsetse control.

For continental database development, a detailed administration boundary layer for Africa has been acquired from the Food and Agriculture Organization (FAO). This layer contains updated boundaries on provincial, district and sometimes sub-district levels. This layer has been used as the framework for developing cattle population size and distribution data for most sub-Saharan countries to the highest level of spatial resolution available in published country reports. The GIS was then utilized to calculate cattle density. A very similar approach has been used at the University of Southern California for human population density estimations for Africa, and this updated (1994) information has been acquired recently.

ILRAD, ICRAF, IITA and the Australian National University (ANU) have jointly collaborated in the development of Africa-wide interpolated climate surfaces at a 4 km resolution. These include long-term monthly mean minimum and maximum temperature, rainfall and elevation, resulting in 37 new data layers. These climate surfaces are at a sufficiently high resolution to be of value for national-level research and decision-support purposes. Previous to this effort, only coarse resolution surfaces (25 km) or meteorological station point data were available. Many other climatic variables important for discrimination of various vector biological requirements have been derived from this monthly surfaces and will be extremely useful for modelling and decision-support systems.

Several other databases were acquired and developed. The World Conservation Monitoring Centre (WCMC) provided update of their Africa-wide protected areas coverage. Development of the digital Kenya human population layers for 1979 and 1989 were completed. Continental data layers (forest, wetlands, desert, wetlands and mangrove) were developed from data published by the World Resources Institute (WRI).

**SEP2.2 Development of decision-support systems  
for the control of animal diseases**

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The objective of this activity is to develop information and decision-support systems targetted at different end-users (such as farmers, dairy societies, private veterinarians, government veterinary services, development planners and donors) for the improved use of available knowledge and technologies in the control of animal diseases. Currently, most emphasis is on public sector users, particularly disease control planners, donors and research organizations, but in late 1994 a research project targetted at the private sector was initiated.

During 1994, a framework decision-support system was established in Zimbabwe for use by researchers and the field services branch of the Department of Veterinary Services. Funding for this activity was provided in part by the Australian Centre for International Agricultural Research (ACIAR). This project is also intended to complement and provide timely information to other related ACIAR projects in Zimbabwe, such as one developing improved methods for diagnosis and control of bovine babesiosis and anaplasmosis.

Custom-designed disease surveillance software was developed at ILRAD specifically for this project. GIS software was specifically chosen for 'suitability to task' and compatibility with other systems currently operational in Zimbabwe. Over 90 georeferenced data layers for the country were developed and incorporated into an integrated animal health decision-support system. These databases include information on the veterinary and country infrastructure, notifiable disease reporting, vector distributions, wildlife distributions, cattle density estimations, demographics, natural resources and climatic variables.

The system is now in active use by the department in the areas of tick-borne diseases, rabies and Newcastle disease control. In late 1994, the system was further developed through the introduction of EPIMAN, a decision-support system for improved control of major infectious diseases, in collaboration with Massey University, New Zealand.

A new collaboration was initiated in 1994 at the Animal Health Research Centre, Entebbe, Uganda. With GTZ funding, an animal health information system using GIS had been established, and the use of this for research and decision support is being

developed in collaboration with ILRAD. Climate databases have been installed by ILRAD, and georeferenced databases on health and productivity in the Ankole Ranching Scheme are being developed.

Also initiated in 1994 was a study to develop decision-support systems for small-holder dairy producers. The intention is to promote better decision making on resource allocation so as to improve milk production through better animal health. This research is being conducted in collaboration with the Department of Farm Management, Wageningen Agricultural University, The Netherlands. In a pilot study, the role of two developed models TIES and TACT, were evaluated in a case study carried out with members of a dairy society in Murang'a District, Kenya.

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**STAFF PRESENTATIONS  
AT INTERNATIONAL MEETINGS**

**A. Andrianarivo**

FASEB Summer Research Conference on Principles in Viral, Bacterial and Protozoan Pathogenesis, Saxtons River, Vermont, USA; 19–24 June 1994. Poster presented: *Kinetics of haemopoietic progenitor cells during a primary Trypanosoma congolense infection in N'Dama and Boran cattle.*

**E. Authie**

International Congress of Parasitology, Izmir, Turkey, 10–14 October 1994. Paper presented: *Expression of the C-terminal and central domains of congopain, a cysteine protease of Trypanosoma congolense, and their immune recognition by cattle of differing susceptibility to trypanosomiasis.* Authié E. and Boulangé A.

**R. Bishop**

Woods Hole Molecular Parasitology Meeting, Woods Hole, Massachusetts, USA, 17–22 September 1994. Paper presented: *Analysis of sexual crosses between Theileria parva stocks.*

Nairobi Cluster Symposium, National Museums of Kenya, Nairobi, Kenya, 23 June 1994. Paper presented: *Nucleic acid probes for Theileria species.*

Onderstepoort Veterinary Institute, Pretoria, Republic of South Africa, 28 November 1994. Paper presented: *Analysis of sexual crosses between Theileria parva stocks.*

**T. Dolan**

OAU/FAO and ILRAD Regional Tick and Tick-Borne Diseases Workshop on Integrated Tick and Tick-Borne Disease Control, Lilongwe, Malawi, 26–29 April 1994. Paper presented: *Status of standards for live tick-borne disease vaccines.*

Third European Union Coordination Meeting on *Theileria annulata*, Anatolia, Turkey, 4–9 October 1994. Paper presented: *Research progress on Theileria parva.*

Nineteenth Session of the FAO Regional Animal Production and Health Commission for Asia and the Pacific (APHCA), Tehran, Iran, 20–24, November 1994. Paper presented: *The International Livestock Research Institute (ILRI) and its role in Asia.*

**L. Gaidulis**

Second African Immunology Conference, Nairobi, Kenya, 23–27 October 1994. Poster presented: *RT-PCR analysis of TNF-alpha and IL-1 mRNA in cattle during African trypanosomiasis.*

**B. King**

South African Association of Laboratory Animals Science, Esselen Park, Johannesburg, Republic of South Africa, 5–7 October 1994. Papers presented:

1. *The duties and role of an institute animal care and use committee.*
2. *The design and use of a large plastic film negative pressure isolator for calves.*

3. *The role of the laboratory animal technician in the management of a large multi-purpose experimental animal facility in Kenya.*

### **C. Laker**

Seventh International Symposium on Veterinary Epidemiology and Economics, Nairobi, Kenya, 15–19 August 1994. Paper presented: *A financial analysis of East Coast fever immunization in Mbarara District, Uganda.* C.D. Laker, A.W. Mukhebi, G.S.Z. Ssenyonga, J.M. Gathuma, Y.K. Ssentongo and C.P. Otim.

### **L. Logan-Henfrey**

Second African Immunology Conference, Nairobi, Kenya, 23–27 October 1994. Poster presented: *Enrichment of bovine haemopoietic progenitor cells.* P. Muiya, L. Logan-Henfrey and J. Naessens.

Second International Cytokine Conference, Banff, Canada, 1–5 October 1994. Poster presented: *Cloning of bovine stem cell factor cDNA.* B. Mertens and L. Logan-Henfrey.

### **V. Lutje**

Second African Immunology Conference, Nairobi, Kenya, 23–27 October 1994. Papers presented:

1. *Longterm bovine immune responses to defined Trypanosoma congolense antigens.* V. Lutje, K. Taylor, A. Boulangé and E. Authié.
2. *In vitro activation and detection of antibody secreting cells from Trypanosoma congolense-infected cattle.* K.A. Taylor, B. Gichuki, V. Lutje, J. Naessens and D.J.L. Williams.

Twelfth European Immunology Meeting, Barcelona, Spain, 14–17 June, 1994. Paper presented: *Bovine T cell responses to Trypanosoma congolense antigens during infection.* V. Lutje, E. Authié, B. Mertens, A. Boulangé and D.J.L. Williams.

### **R. Masake**

VIII International Congress of Parasitology, Izmir, Turkey 10–14 October, 1994. Paper presented: *Characterization of trypanosome circulating antigen.*

Twenty-third Annual Congress of Parasitology Society of Southern Africa, 30 June to 1 July, 1994. Paper presented: *Characterization of Trypanosoma vivax antigen recognized by the species-specific monoclonal antibody employed in the antigen-detection ELISA.*

### **D. McKeever**

Cold Spring Harbour Vaccines Meeting, Cold Spring Harbour, USA, October 5–9, 1994. Paper presented: *Epitope specificity of bovine immune responses to the major surface protein of Theileria parva sporozoites.*

**B. Mertens**

Second International Cytokine Conference, Banff, Canada, 1–5 October, 1994. Poster presented: *Cloning of bovine stem cell factor cDNA*. B. Mertens and L. Logan-Henfrey.

**S. Morzaria**

British Society for Parasitology's Sixth Annual Malaria Meeting, Liverpool, UK, September 1994. Paper presented: *Analysis of sexual crosses between Theileria parva stocks*.

**A. Mukhebi**

Symposium on Agricultural Policies and Food Security in Eastern and Southern Africa Region, The Kenya Commercial Bank Training Institute, Karen, Nairobi, Kenya, 18–20 May 1994. Paper presented: *Animal disease research for food security in sub-Saharan Africa: The case of theileriosis and trypanosomiasis in the Preferential Trade Area*. A.W. Mukhebi, B.D. Perry and R. Kruska.

Twelfth International Conference of Agricultural Economists, Harare, Zimbabwe, 22–26 August 1994. Paper presented: *Livestock diseases and food security in the Preferential Trade Area with a focus on Zimbabwe*. A.W. Mukhebi, T. Munyombwe, C. Ncube, R.L. Kruska, U. Ushewokunze-Obatolu, C. Bamhare and B.D. Perry.

Fourth KARI Scientific Conference, Nairobi, Kenya, 25–28 October, 1994. Paper presented: *Systematic approach towards delivery of East Coast fever immunization to small-holder dairy farms: the coastal Kenya experience*. S.H. Maloo, D.P. Kariuki, G. Kioo, S.K. Mbogoh, B.D. Perry and W. Thorpe.

Seventh International Symposium on Veterinary Epidemiology and Economics, Nairobi, Kenya, 15–19 August 1994. Paper presented: *Comparative regional assessment of the economic impact of theileriosis and its control in Africa*. A.W. Mukhebi, B.D. Perry, C.D. Laker, D.G. Onchoke, T. Munyombwe and Z.S. Hassan.

**A. Musoke**

Expert Consultation on Tick-Borne Diseases of Sheep and Goats, FAO, Rome, 28–30 September, 1994. Paper presented: *Sub-unit vaccines for the control of tick-borne diseases: implications for the future*. A.J. Musoke, D.M. McKeever and V. Nene.

**J. Naessens**

Twelfth European Immunology Meeting, Barcelona, Spain, 14–17 June, 1994. Posters presented:

1. *T cell depletions in cattle infected with Trypanosoma congolense*. J. Naessens, J.P.Y. Scheerlinck and M. Sileghem.
2. *Induction of TNF- $\alpha$  during trypanosomiasis*. M. Sileghem, L. Gaidulis and J. Naessens.

### **V. Nene**

Cold Spring Harbour meeting on Ribosome synthesis and Nucleolar function, Cold Spring Harbour, USA, 28 September to 2 October, 1994. Poster presented: *A 7.1 kbp extrachromosomal DNA molecule of Theileria parva has scrambled rDNA sequences and encodes mitochondrial proteins*. V. Nene, A. Kairo, E. Gobright, A. Fairlamb and G. Shukla.

### **A. Peregrine**

Annual Scientific Conference of the Veterinary Association, Nairobi, Kenya, April, 1994. Paper presented: *Occurrence and control of multiple-drug resistance trypanosome infection in cattle at Ghibe, Ethiopia*. A.S. Peregrine, W. Mulatu, S.G.A. Leak and G.J. Rowlands.

Seventh International Symposium of Veterinary Epidemiology and Economics, Nairobi, Kenya, August, 1994. Paper presented: *Epidemiology of bovine trypanosomiasis in the Ghibe valley, Ethiopia: multiple-drug resistance and its effective control*. A.S. Peregrine, W. Mulatu, S.G.A. Leak, and G.J. Rowlands.

Seminar to the Department of Veterinary Research, Harare, Zimbabwe, November 1994. Paper presented: *Epidemiology of bovine trypanosomiasis in the Ghibe valley, Ethiopia: multiple-drug resistance and its effective control*. A.S. Peregrine, W. Mulatu, S.G.A. Leak, and G.J. Rowlands.

### **B. Perry**

Workshop on the role of epidemiology in animal health research, NVRC, 14 March, 1994, Kenya Agricultural Research Institute, Muguga, Kenya. Paper presented: *The role of epidemiology in animal health research*.

Workshop on generic decision support systems for livestock disease control, Aberdare Country Club, Kenya, 5–7 May 1994. Paper presented: *ILRAD and decision-support systems*.

WHO Consultation on Development and Application of Geographical Methods in the Epidemiology of Zoonoses, Held in the Federal Research Centre for Virus Disease of Animals, Institute of Epidemiology, Wusterhausen, Germany, 30 May–2 June 1994. Paper presented: *Predicting the distributions of Rhipicephalus appendiculatus and other tick vectors in Africa using geographical information systems*.

Workshop to Coordinate Studies of Land-use Change in the Zambezi Valley of Zimbabwe. Paper presented: *Collaborative studies on the impact of tick-borne disease control in Zimbabwe*.

Course held in association with the International Symposium on Veterinary Epidemiology and Economics, Nairobi, Kenya, 8–13 August, 1994. Paper presented: *Disease control programmes in developing countries: prospects and constraints*.

Seventh International Symposium on Veterinary Epidemiology and Economics, Nairobi, 15–19 August 1994. Paper presented: *The role of epidemiology and economics in the control of tick-borne diseases of livestock in Africa*.

**R. Reid**

Workshop entitled *Land-use Change: The Link Between Human-Dominated Systems and Biodiversity in Africa*, Nairobi, Kenya, 20–23 July, 1994. Paper presented: *Biodiversity in Africa's human landscapes*.

Annual Meeting of the Ecological Society of America, Knoxville, Tennessee, USA, 31 July–4 August, 1994. Paper presented: *Ecological consequences of controlling a livestock disease: changes in land-use and vegetative cover in Ethiopia*. R.S. Reid, C.J. Wilson, R.L. Kruska and B.M. Swallow.

Seventh International Ecology Congress, Manchester, England, 20–26 August, 1994. Paper presented: *Trypanosomiasis control: The conflict between food production and biodiversity in Africa*. R.S. Reid, R.L. Kruska, C.J. Wilson, B.M. Swallow, J.E. Ellis and B.D. Perry.

**M. Sileghem**

Twelfth European Immunology Meeting, Barcelona, Spain, 14–17 June 1994. Poster presented: *Induction of TNF during trypanosomiasis*. M. Sileghem, L. Gaidulis and J. Naessens.

**K. Taylor**

Second African Immunology Conference, Nairobi, Kenya, 23–27 October 1994. Poster presented: *In vitro activation and detection of antibody secreting cells from Trypanosoma congolense antigens*. V. Lutje, K. Taylor, A. Boulangé and E. Authié.

**A. Teale**

European Bovmap Meeting, Soria Moria Conference Centre, Oslo, Norway, 4–5 February 1994. Paper presented: *Bovine gene mapping in Africa*.

Fifth World Congress on Genetics Applied to Livestock Production, University of Guelph, Guelph, Ontario Canada, 7–12 August 1994. Paper presented: *Conventional and molecular immunogenetics: potential impact on livestock improvement*.

FAO Symposium on Conservation of Domestic Animal Diversity at the Fifth World Congress on Genetics Applied to Livestock Production, University of Guelph, Guelph, Ontario, Canada, 11 August 1994. Paper presented: *Applications of molecular genetic and reproductive technologies in the conservation of domestic animal diversity*. A.J. Teale, S.G. Tan and J-h. Tan.

Seventh International Symposium on Veterinary Epidemiology and Economics, Nairobi, Kenya, 15–19 August 1994. Paper presented: *Molecular genetic characterization of animal species, with particular reference to disease resistance and susceptibility*.

**C. Wells**

Thirty-third Annual Congress of the Electron Microscopical Society of Southern Africa (EMSSA) held at the University of Port Elizabeth, Eastern Cape, Republic of

South Africa. Paper presented: *Localization of Samorin in Trypanosoma congolense by fluorescence immunoelectron microscopy and autoradiography*. C. Wells, J. Wilkes and A.S. Peregrine.

**A. Young**

Fifteenth African Health Sciences Congress, Nairobi, Kenya, 11–14 February, 1994. Paper presented: *Biological response modifiers: cytokines in health and disease and their possible therapeutic value*. A.S. Young and D. Koech.

OAU/FAO and ILRAD Regional Tick and Tick-Borne Diseases Workshop on Integrated Tick and Tick-Borne Disease control, Lilongwe, Malawi, 26–29 April, 1994. Paper presented: *Diapause in Rhipicephalus appendiculatus*.

Nairobi Cluster Symposium—New Diagnostic Methods for Parasitic Infections in Humans and Animals and Their Application, National Museums of Kenya, Nairobi, Kenya, 23 June, 1994. Paper presented: *Requirements of new diagnostic tests for animals*.

Seventh International Symposium on Veterinary Epidemiology and Economics, Nairobi, Kenya, 15–19 August 1994. Papers presented:

1. *The biology of the transmission dynamics of Theileria parva*. A.S. Young, H. Ochanda, B.D. Perry, S.P. Morzaria, T.T. Dolan, G.F. Medley and G. Gettinby.
2. *Analysis of longitudinal serological data for Theileria parva*. C.J. O'Callaghan, J.J. McDermott, J.M. Katende, J.E. Adams, B.D. Perry and A.S. Young.

Third International Conference on Tropical Entomology, Nairobi, Kenya, 30 October–4 November, 1994. Papers presented:

1. *Ixodid ticks as vectors of parasitic diseases and their population management*.
2. *Prospects for investigating tick pathogen relationships using artificial feeding systems: a case of Rhipicephalus appendiculatus*. S.M. Waladde, A.S. Young, S.N. Mwaura, G.N. Njihia and F.N. Mwakima.
3. *Acaricidal activity of extracts from Melia volkensii, Azadiradta indica and Boophilus decoloratus*. M. Mwikali, R. Mwangi, A.S. Young and J.M. Kabaru.

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# SHORT-TERM VISITING SCIENTISTS

Name	Project	Dates
Prof. V. Anosa University of Ibadan Nigeria	Anaemia of trypanosomiasis	19/9/93–1/4/94
Dr. I. McKendrick University of Strathclyde Glasgow, Scotland	Generic decision support systems	15/11/93–4/1/94 20/4/94–16/5/94 26/11/94–12/12/94
Dr. M. Desquesnes CIRAD/EMVT-Guyane French Guyana	PCR for improved trypanosome diagnosis in South America	4/2/94–22/4/94
Dr. G. Schares Free University of Berlin Germany	Collaborative studies on trypanosomiasis epidemiol- ogy and chemotherapy	13/2/94–21/2/94
Dr. R. Brun Swiss Tropical Institute Switzerland	Collaborative studies on trypanosomiasis chemotherapy	18/3/94–8/4/94
Dr. R. Pegram Regional Coordination and Training Project FAO, Zimbabwe	Planning discussions for Malawi Tick Borne Diseases Workshop	11/4/94–14/4/94
Dr. A. Permin FAO, Phase III Regional Programme Rome, Italy	Planning discussions for Malawi Tick-Borne Diseases Workshop	11/4/94–14/4/94
Ms. Yiqun Gu University of Strathclyde Glasgow, Scotland	Generic decision-support systems	20/4/94–16/5/94 26/11/94–12/12/94
Dr. D. Rebeskie IAEA, Vienna Austria	Antigen detection systems for trypanosomes	23/4/94–3/6/94
Prof. G. Gettinby Strathclyde University Glasgow, Scotland, UK	Generic decision- support systems	2/5/94–15/5/94 29/11/94–10/12/94
Dr. Crawford Revie Strathclyde University Glasgow, Scotland, UK	Generic decision- support systems	2/5/94–15/5/94 29/11/94–10/12/94

**SHORT-TERM VISITING SCIENTISTS**

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<b>Name</b>	<b>Project</b>	<b>Dates</b>
Dr. M. Szulczynski Kenyatta University Nairobi, Kenya	Genetic basis of resistance to isometami- dium in trypanosomes	1/3/94–31/5/94
Dr. C. Sugimoto Hokkaido University Sapporo, Japan	Epidemiology of theileriosis	24/6/94–1/7/94
Dr. N. Tsuji JIRCAS/TARC Tsukuba, Japan	Expression of HSP proteins during theilerial infection	28/6/94–20/8/94
Dr. J. Ellis Winrock International Morrilton, Arkansas, USA	Economics of disease	8/7/94–20/7/94
Dr. D. Zilberstein Israel Institute of Technology Haifa, Israel	Drug transport by trypanosomes	26/7/94–23/8/94
Dr. R. MacFarlane Lincoln University Canterbury, New Zealand	Helminth Immunology (ILCA Visiting Scientist)	1/8/94–31/3/95
Dr. A. Lew Animal Research Institute Queensland, Australia	DNA characterization of haemoparasites	13/8/94–16/9/94
Dr. Brenda Harold Brunel University Bristol, England	Supervision of Ph.D. students	30/8/94–5/9/94
Dr. G. Russell Roslin Institute Edinburgh, Scotland, UK	Bovine MHC typing techniques	5/9/94–4/12/94
Prof. S. Aksoy Yale University New Haven, CT, USA	Tsetse symbiosis research	10/9/94–20/9/94
Prof. A. Dijkhuizen Wageningen University The Netherlands	Consultant Farming systems	8/10/94–15/10/94

Name	Project	Dates
Dr. Annette Gelhaus Bernhard Nocht Inst. Hamburg, Germany	Genome mapping	8/10/94–23/10/94
Dr. R. Horstmann Bernhard Nocht Inst. Hamburg, Germany	Genome mapping	8/10/94–23/10/94
Prof. J. Mansfield Univ. of Wisconsin Madison, USA	Trypanosome immunology	26/10/94–30/10/94
Prof. K.H. Zessin Free University of Berlin Germany	Collaborative studies on trypanosomiasis epide- miology and chemotherapy	21/11/94–22/11/94
Dr. D. Pfeiffer Massey University Palmerston New Zealand	Consultant GIS decision- support systems	21/11/94–8/12/94
Dr. T. Chamboko Heartwater Research Project VRL, Harare, Zimbabwe	Collaborative studies on heartwater research	16/10/94–23/10/94
Dr. A.J. Van der Zijpp IVO-DLO Research Institute for Animal Production Leleystad, The Netherlands	Collaborative research on trypanosomiasis chemo- therapy/biochemistry	21/9/94–23/9/94
Dr. D. Parkin Department of Chemistry Chestnut Hill College Philadelphia, USA	Trypanosome nucleoside hydrolase in susceptibility to samorin	27/8/94–26/7/95
Dr. Mara Rocchi University of Milan Italy	Activation of cytokine production by bovine macrophages	30/7/94–1/2/95
Dr. P. Totté iEMVT Maisons Alfort, France	Analysis of the basis of protection in cattle with killed <i>Cowdria ruminantium</i>	8/3/94–30/9/95

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# ABBREVIATIONS, ACRONYMS AND SYMBOLS

ACP	acid phosphatase
ALVC	afferent lymph veiled cells
ASC	antibody secreting cells
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
AuTat	Antwerp <i>Trypanozoon</i> antigenic type
B-cells	A class of lymphocytes which can be stimulated to produce antibodies
BFA	Bafilomycin A1
BFU-E	burst-forming units-erythroid. Colonies of early red blood cell precursors which arise in some bone marrow cultures
BMMNC	bone marrow mononuclear cells
BiP	Immunoglobulin binding protein
BPV	bovine papilloma virus
BSA	bovine serum albumin
BSFs	bloodstream forms (of trypanosomes)
<sup>14</sup> C	a radioactive isotope of carbon
C-terminal	The carboxy-terminus of a protein or peptide
cDNA	complementary deoxyribonucleic acid
CD	curative dose
CD5+	a leukocyte surface antigen predominantly associated with a subclass of B cells
CD-ROMs	compact disks-read only memory
CFU-E	colony-forming units-erythroid. Colonies of late red blood cell precursors which arise in some bone marrow cultures
CFU-GM	colony-forming units, granulocyte/macrophage progenitors
CFU-S	pluripotent stem colonies
CGIAR	Consultative Group on International Agricultural Research (Washington, DC)
CKII	casein kinase II
CLA	complement lysis assay
"congopain"	a cysteine protease purified from <i>T. congolense</i>
<sup>51</sup> Cr	a radioactive isotope of chromium
CSIRO	Commonwealth Scientific and Industrial Research Organization (Australia)
CTLp	cytotoxic T-lymphocyte precursor(s)
DA	diminazene aceturate
DEAE	diethylaminoethyl (cellulose). A positively charged chromatography resin
DFMO	DL- $\alpha$ -difluoromethylornithine
DFP	diisopropylfluorophosphate
DNA	deoxyribonucleic acid
dpi	day(s) after infection
dT	deoxythymidine
E-64	a protease inhibitor specific for cystein proteases

ABBREVIATIONS, ACRONYMS AND SYMBOLS

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EC <sub>50</sub>	the concentration of drug in culture that reduces parasite growth by 50% compared to control cultures
ECF	East Coast fever
EcoRI	a restriction enzyme (which cuts nucleotide sequences)
ER	endoplasmic reticulum
FAO	Food and Agriculture Organization of the United Nations (Rome)
FBS	foetal bovine serum
FFE	free-flow electrophoresis
FITC	fluorescein isothiocyanate
Forest-type	a sub-specific group of genetically similar <i>Trypanosoma congolense</i> parasites identified by a repetitive sequence DNA probe
g	gramme
GAC	global area coverage
GIS	geographic information system(s)
GRID	Global Resource Information Database (United Nations Environment Programme, Nairobi)
GuTat	Glasgow University <i>Trypanozoon</i> antigenic type
HCT	haematocrit centrifuge technique
Hind III	a restriction endonuclease
HMI-93	a liquid medium for supporting trypanosome growth <i>in vitro</i> (devised by H. Hirumi and colleagues at ILRAD)
hr	hours
HRP	horseradish peroxidase
HSP	heat shock protein
HSS	high-speed supernatant
<sup>125</sup> I	a radioactive isotope of iodine
IAEA	International Atomic Energy Agency (Vienna)
IFAT	indirect fluorescent antibody test
Ig	immunoglobulin
IgG <sub>1</sub> /IgG <sub>2</sub>	immunoglobulin gamma, classes 1 and 2
IL-2R	Receptor for interleukin-2
ILDat	ILRAD <i>Duttonella</i> antigenic type
ILNat	ILRAD <i>Nannomonas</i> antigenic type
ILTat	ILRAD <i>Trypanozoon</i> antigenic type
ILCA	International Livestock Centre for Africa (Addis Ababa)
ILRAD	International Laboratory for Research on Animal Diseases (Nairobi)
IM	intramuscular
iso-VAT(s)	antigenically similar variable antigen type(s) of trypanosomes
IV	intravenous
IVDST	<i>in vitro</i> drug sensitivity test
K <sup>+</sup>	potassium ion
KARI	Kenya Agricultural Research Institute
Kd	dissociation constant
KETRI	Kenya Trypanosomiasis Research Institute
kg	kilogramme

$K_i$	inhibition constant
Kilifi-type	a sub-specific group of genetically similar <i>Trypanosoma congolense</i> parasites identified by a repetitive sequence DNA probe
km	kilometre
Km	rate constant
LD50	the dose at which 50% of animals are killed (50% lethal dose)
LN	lymph node
LNC	cells derived from lymph node tissue
LPS	lipopolysaccharide(s)
LR	Land Registration (unit[s]) (Uasin Gishu District, Kenya)
M	molar
MAb(s)	monoclonal antibody(ies)
MCHC	mean cell haemoglobin concentration
MCV	mean cell volume
MCFs	metacyclic forms (of trypanosomes)
mdr	a transcribed gene whose increased expression is specific to multidrug resistance cell lines
MDR	multidrug resistance
medRNA	mini-exon-derived ribonucleic acid
Mel B	melarsoprol
MEM	modified Eagle's medium
mg	milligramme
min	minute(s)
ml	millilitre
mM	millimolar
MO	monocyte(s)
mol	mole
mRNA	messenger ribonucleic acid
MW	molecular weight
mVAT(s)	metacyclic variable antigen type(s)
N-terminal	The amino terminus of a protein or peptide
$Na^+$	sodium ion
NAC	non adherent cells
NaOH	sodium hydroxide
NDVI	normalized difference vegetation index
NEM	N-ethylmaleimide
ng	nanogramme
NIT	neutralization of infectivity test
NotI	a rare cutting restriction endonuclease
nm	nanometre
nmole	nanomole (10 <sup>-9</sup> of a mole)
OAU	Organization of African Unity (Addis Ababa)
OD	optical density (e.g. of the colour reaction in an ELISA)
ODA	Overseas Development Administration (UK)
OFAGE	orthogonal field alternation gel electrophoresis
ORF(s)	open reading frame(s) (i.e. potentially transcribed length of DNA)

ABBREVIATIONS, ACRONYMS AND SYMBOLS

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p85	an 85-kilodalton protein antigen on the surface of <i>Theileria parva</i> sporozoites and schizonts
PCV(s)	packed cell volume(s) (percent)
PFR	paraflagellar rod - an organelle in the flagellum of trypanosomes
pH	hydrogen ion concentration
pi	post infection
pI	isoelectric point
poly(A) <sup>+</sup> RNA	polyadenylic acid residues in messenger RNA, usually marking one end of the message
pMAL	a vector for the expression of introduced genes as proteins
pMAL-P	a protein expression system producing the protein expressed to a carrier which can be subsequently cleaved by the protease factor Xa
pmole	picomole ( $10^{-12}$ of a mole)
PWM	pokeweed mitogen
RAPD	randomly amplified polymorphic DNA
RBC	red blood cell(s)
RFLP	restriction fragment length polymorphism
rIL	recombinant interleukin
rRNA	ribosomal ribonucleic acid
RNA	ribonucleic acid
S	Svedberg unit
SI	stimulation index
ss-cDNA	single stranded, complementary deoxyribonucleic acid
Savannah-type	a sub-specific group of genetically similar <i>Trypanosoma congolense</i> parasites identified by a repetitive sequence DNA probe
SEAP	secreted placental alkaline phosphatase
SIT	Sterile Insect Technique
T-plasmid vectors	a particular type of DNA plasmid useful for cloning polymerase chain reaction products
TcCCP	<i>Trypanosome congolense</i> cysteine protease precursor
TCR	T-cell receptor(s)
TCGF	T-cell growth factor
Tf-R	transferrin receptor
tRNA	transfer ribonucleic acid
Triton X-100	a detergent
Tsavo-type	a sub-specific group of genetically similar <i>Trypanosoma congolense</i> parasites identified by a repetitive sequence DNA probe
SfiI	a rare-cutting restriction endonuclease
UNDP	United Nations Development Programme (New York)
UTR	untranslated region (of a gene sequence)
VAT(s)	variable antigen type(s) (of trypanosomes)
VSG	variable surface glycoprotein(s) (of trypanosomes)
v/v	volume per volume
WHO	World Health Organization (Geneva)
Xa	a protease

xg	gravity
XhoI	a restriction enzyme
$\mu$	micro ( $10^{-6}$ )
$\mu$ g	microgramme ( $10^{-6}$ of a gramme)
$\mu$ M	micromolar
$\alpha$	alpha
$\alpha$ 2M	alpha-2-macroglobulin, a serum protease inhibitor
$\beta$	beta
$\lambda$	lambda
$\lambda_{\text{max}}$	maximum wavelength
$\mu$ l	microlitre
$\lambda$ gt11	an engineered bacteriophage for use in cloning DNA sequences
$^{\circ}$ C	degrees Celsius
10-mers (etc)	sequences of DNA containing 10 nucleotides etc.