NOVEL IMMUNIZATION STRATEGIES AGAINST PROTOZOAN PARASITES

PROCEEDINGS OF A WORKSHOP HELD AT ILRAD

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THE INTERNATIONAL LABORATORY FOR RESEARCH ON ANIMAL DISEASES

BOX 30709 . NAIROBI . KENYA

The International Livestock Research Institute (ILRI) began operations on 1 January 1995. The institute incorporates the resources, facilities and major programmes of two former CGIAR centres founded about two decades ago-the International Laboratory for Research on Animal Diseases, in Nairobi, Kenya, and the International Livestock Centre for Africa, in Addis Ababa, Ethiopia. The research and outreach programmes of ILRAD and ILCA have been consolidated, streamlined and reoriented to support an expanded mandate. ILRI will conduct strategic research in the biological, animal and social sciences to improve livestock productivity in sustainable agricultural systems throughout the developing world.

The objectives of the new institute's research programme are to improve animal health, nutrition and productivity (milk, meat, traction) in ways that are sustainable over the long term, to characterise and conserve the genetic diversity of indigenous tropical forage species and livestock breeds, to promote sound and equitable national policies for animal agriculture and natural resource management, and to strengthen the animal husbandry research programmes of developing countries.

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Foreword

The International Laboratory for Research on Animal Diseases (ILRAD) was established in 1973 by the Consultative Group on International Agricultural Research (CGIAR) and given a mandate to conduct intensive research leading to the improved control of important livestock diseases in developing countries. Research at ILRAD concentrated initially on developing novel immunological control methods for theileriosis and trypanosomiasis. More recently the Theileriosis Programme has been expanded to cover other tick-borne diseases.

East Coast fever is a virulent and usually fatal disease caused by the parasite *Theileria parva*. It is estimated that about 40% of the 63 million cattle in eastern, central and southern Africa are at risk of infection with the organism. *Theileria parva* is an intracellular protozoan parasite that invades bovine lymphocytes, causing them to proliferate in an uncontrolled manner. Intense destruction of lymphoid organs coupled with invasion of non-lymphoid tissues with parasitized cells lead ultimately to the death of the majority of infected cattle. Those animals that recover from infection are solidly immune to homologous challenge, and a method of vaccination is available based on infection with a sporozoite stabilate in the face of treatment with long acting formulations of oxytetracycline. Major drawbacks of this form of immunization are its dependence on an effective cold chain, and the considerable antigenic diversity that exists between different isolates of the parasite. The development of a broadly protective subunit vaccine against *T. parva* has therefore been a major research goal of ILRAD's Tick-Borne Diseases Programme.

Bovine trypanosomiasis affects some 50 million cattle in Africa and is considered to be the major disease constraint on livestock production in sub-Saharan Africa. The disease is caused by an extracellular protozoan parasite and is characterized by anaemia, progressive cachexia and eventual death. The disease has serious effects on productivity and reduces the reproductive potential of the host. The absence of sterile immunity following trypanosome infection due to the parasite's ability to vary its surface glycoprotein coat led to concern that a vaccine against trypanosomiasis may not be practical. However this view has been overturned by recent findings. Certain breeds of cattle, found mainly in West Africa, have evolved a degree of genetic resistance to the disease and are termed trypanotolerant. Trypanotolerant cattle appear to control parasite numbers as well as the pathological consequences of the disease. The description and characterization of trypanotolerance has renewed interest in the feasibility of designing novel immunological control methods for the disease. The trypanosomiasis research programme at ILRAD is now largely focused on the elucidation of mechanisms responsible for trypanotolerance. Its major goals are to determine if there is an immunological basis for trypanotolerance, to ascertain how trypanotolerant cattle control their parasitaemia and to identify the genes responsible for the trait.

The parasite systems in which ILRAD is involved are complex and present problems similar to those faced by scientists attempting to develop improved control measures against malaria, leishmaniasis and Chaga's disease. Much information has been generated in the latter systems that have altered current perceptions of the role of immunity in protection and disease and modified

vaccine development strategies. It was therefore considered timely that a workshop be convened to discuss novel immunization strategies against protozoan parasites. The principal aim of the meeting was to review those developments in the fields of immunology and immunoparasitology that have relevance to the development of vaccination strategies for ECF and trypanosomiasis. Of relevance to the control of ECF were recent developments in the elucidation of events that surround the induction of cytotoxic T lymphocytes, and the role of cytokines in the control and pathogenesis of parasitic infections. Chief areas of interest to the trypanosomiasis programme were disease-related perturbations of the immune system, the role of individual T cell subsets in immunity to parasites and the involvement of cytokines in the control and pathogenesis of parasitic diseases. More specific discussions focused on:

- the role of CTL in parasitic infections;
- antigen processing for class I MHC-restricted responses;
- novel technologies for identifying antigens that provoke CTL responses;
- the role of detrimental immune responses in pathogenesis;
- the role of T cell subsets and their products in the control of parasitic diseases; and
- immunization strategies for driving immune responses towards favourable rather than detrimental mechanisms.

Ten experts in these fields participated in the workshop, and through lively discussions contributed to be formulation of a series of recommendations that will be incorporated in ILRAD's immunology programmes for the next five years.

D.J.L. Williams
Chairperson, Workshop Organizing Committee
International Laboratory for Research on Animal Diseases
Nairobi, Kenya

January, 1994.

Workshop Organizing Committee

Eley, R.
McKeever, D.J.
Naessens, J.
Sileghem, M.
Williams, D.J.L.

INTRODUCTION TO ILRAD'S RESEARCH PROGRAMMES

The Trypanosomiasis Research Programme at ILRAD

A.J. Teale

International Laboratory for Research on Animal Diseases
P.O. Box 30709, Nairobi, Kenya

Research into improved means of control of trypanosomiasis of livestock has been a part of the Laboratory's research agenda since its inception in the 1970s. At that time, it was recognized that trypanosomiasis constituted the most significant disease constraint on livestock development in sub-Saharan Africa. Also at that time, rapid advances were being made in understanding the basis of immune responses and in the development of new vaccines. Twenty years later, trypanosomiasis remains the most important disease constraint on livestock development on the African continent, and to a lesser, but still important extent, in other parts of the world where non-tsetse transmitted forms of the diseases are prevalent. This is particularly the case in Asia and Latin America. It is difficult io ascertain the real costs of the disease in direct and indirect terms, but a generally accepted figure is $5 billion annually in sub-Saharan Africa alone. The disease also affects man, and the number of new cases reported each year runs into the tens of thousands, many of which prove fatal.

The trypanosomiasis research programme in the Laboratory has two broad objectives. First, to develop approaches and technologies which will enable currently available control strategies to be applied more effectively and, second, to develop novel means of control. Trypanosomiasis control at present relies on three basic approaches: (a) chemotherapy and chemoprophylaxis, (b) vector-control and (c) the farming of trypanotolerant livestock. The Laboratory has not engaged in research on improved means of vector control, this being the mandate of other institutions, most notably the International Centre for Insect Physiology and Ecology (ICIPE) in Nairobi.

In support of current control options, the Laboratory has placed considerable emphasis on the development of effective diagnostic tools to underpin disease and disease control monitoring. Emphasis has been given to antibody-based systems for detecting circulating parasite antigens and these new and sensitive diagnostic tests have been transferred to the International Atomic Energy Agency for field validation across Africa.

In recent years, reports of apparent drug resistance have been increasing. Given the importance of drugs in the disease controllers' armamentarium, and the fact that all of the currently available drugs are chemically related one to another, the emergence of drug resistance in trypanosomes is a serious concern. The programme at ILRAD has therefore included research into the evolution of drug resistance in the field, on the one hand, and into the molecular basis for drug resistance, on the other. Through better understanding of the circumstances

which lead to the development of drug resistance, and with the availability of rapid and accurate methods to detect and quantify drug resistance in field isolates, it is hoped that chemotherapy can be sustained as an effective control measure into the foreseeable future without the need to develop new drugs-the latter being a very costly and unattractive proposition for the drug industry.

With respect to trypanotolerant livestock, research on the definition of the trypanotolerance trait, estimation of heritability and development of effective means for selection of better trypanotolerant cattle has been undertaken jointly by ILRAD and the International Livestock Centre of Africa (ILCA). This research has led to the identification of selection criteria and development of selection programmes based on field challenge and monitoring of response in immature animals.

The Laboratory's programme on development of novel control strategies envisages two principal outputs-vaccines and candidate trypanotolerance genes.

With respect to vaccine development, scientists at ILRAD, during its early years were instrumental in the description of antigenic variation and subsequently in the description of the genetic control of this phenomenon. The more that was understood about antigenic variation, the clearer it became that vaccines based on immune responses to the variant surface coat of trypanosomes would be unlikely to have an impact in the field. However, increased understanding of the cell biology of trypanosomes and their molecular biology has led to the identification of parasite components and products which may in their own right be responsible for development of disease. This in turn has brought the possibility of development of vaccines based on immune responses to invariant molecules such as cystein proteases and cyclophilins, which are produced by trypanosomes and which may be expected to contribute to the disease process. A further potential option for development of novel strategies based on the immune response is the development of vaccines using invariant antigens of the trypanosome which may be accessible to the humoral arm of the immune system. Work continues in this area.

This research at ILRAD, which may lead to the development of novel control technologies, was considerably strengthened during the 1980s by the establishment of a herd of trypanotolerant N'Dama cattle in the Laboratory, providing scientists with the means to compare and contrast immune and pathological responses in resistant and susceptible host genotypes, and also to compare trypanosome growth and differentiation in the different host types. In this way it may be possible to identify the key features of response to infection which impart resistance to the disease as it occurs in genetically resistant cattle, and, at the same time, this may lead to the identification of candidate genes for the trypanotolerance trait.

During the last few years, a second approach to the identification of genetic regions, and ultimately genes, responsible for the trypanotolerance trait has been developed in the Laboratory. This has involved the breeding of a trypanotolerant resource population based on a cross between tolerant N'Dama cattle and susceptible Boran cattle. Currently, an F2 population is undergoing phenotyping. Using the rapidly developing map of the bovine genome, it will soon be possible to undertake a linkage analysis and search for trypanotolerance control regions in the N'Dama genome. If successful, this will provide animal breeders with markers of the trypanotolerance trait which could be used to introgress resistance genes into highly productive but susceptible genotypes, and

it may be expected to give livestock geneticists essential clues as to where to begin the search for the individual genes. If such genes can be identified, it may be expected that the development of transgenic cattle will follow relatively quickly, but, perhaps even more importantly, an understanding of the molecular bases of this disease resistance trait should be achieved.

It seems certain that trypanosomiasis control in the future, as in the past, will depend on successful integration of the different options available, and the Laboratory will therefore continue to research questions related to optimizing integration of control strategies, whilst at the same time continuing to strive to develop new options to replace or add to those currently available.

The Tick-Borne Diseases Research Programme at ILRAD

T.T. Dolan

International Laboratory for Research on Animal Diseases
P.O. Box 30709, Nairobi, Kenya

The Tick-Borne Diseases Programme at the International Laboratory for Research on Animal Diseases (ELRAD) has as its major objective the improvement of the control of tick-borne diseases by immunological means. The focus of its research has been a novel vaccine for *Theileria parva,* the cause of East Coast fever (ECF) in 11 countries in eastern, central and southern Africa. Conventional control of tick-borne diseases in this region is by strict tick control using regular application of acaricides in dips or sprays. This method has become less reliable because of the development of acaricide resistance, poor management, high cost of acaricides in hard currency, civil unrest and a growing concern about the use of environmentally damaging chemicals. Live vaccines exist for the complex of tick-borne diseases that occur in association with ECF, anaplasmosis, babesiosis and cowdriosis. However, live vaccines have the important limitations of cold chain delivery, induction of persistent infections and the risk of causing diseases during immunization or of introducing pathogens that contaminate the vaccines during preparation. In addition, the live vaccine for *T. parva,* based upon ground up infected tick stabilate inoculated simultaneously with tetracycline, is complicated by a multitude of different antigenic parasite types.

ILRAD's approach to the development of a new vaccine against *T. parva* has been through exploring the protective immune responses in cattle. This work has identified $CD8^+$ T-cell responses directed against parasite antigens on the surface of infected lymphocytes as the major response. In addition, hyperimmune cattle sera and monoclonal antibodies have been shown to neutralize the infectivity of *T. parva* sporozoites, the tick-derived stage inoculated during feeding that invades and transforms lymphocytes. Pursuing this neutralization has identified a dominant circumsporotozite antigen of 67 kDa. The gene for this antigen has been cloned and expressed. Immunization experiments with recombinant p67 have provided infection blocking protection varying from 70-100% and its potential as a future vaccine antigen is being explored. In addition, ILRAD's work has attempted to develop characterization reagents that can distinguish different *Theileria* parasites isolated from the field. This latter research has yielded a range of monocional antibodies and DNA probes that provide unique markers for individual *T. parva* parasites but do not identify its antigenic nature. These reagents are excellent epidemiological and biological tools and have been used to show that many, if not all, field isolates contain mixtures of parasites and that *T. parva* undergoes sexual reproduction which may further contribute to its antigenic complexity. An important research

objective is to complete the development of these technologies as reliable and robust diagnostic and epidemiological tools for the complex of tick-borne diseases.

The search for schizont antigens will be concentrated on the isolation of these antigens using peptide stripping and screening of random DNAs from parasite libraries expressed in COS cells. The focus of research will be maintained on *T. parva*, exploring adjuvant and live vaccination systems, but will be expanded in collaboration with other laboratories working on *Anaplasma, Babesia and Cowdria* to exploit ILRAD's immunological and vaccine development expertise to investigate the vaccine potential of candidate antigens of the other parasites. The long-term objective is the development of antigen-based vaccines for the major tick-borne diseases that can be designed to protect cattle in particular challenge environments. These vaccines will be applied where the tick-borne disease epidemiology has been defined so that the most appropriate control strategy is used.

CLASS I MHC-RESTRICTED RESPONSES IN PARASITIC DISEASES

The role of class I MHC-restricted T-cell responses in immunity to *Theileria parva:* prospects for a vaccine

D.J. McKeever, E.L.N. Taracha, E. Innes , N. MacHugh, H. Goddeeris[†] and W.L. Morrison[‡]*

*Moredun Research Institute, Edinburgh, UK

[†]Catholic University of Louvain, Louvain, Belgium

[‡]Institute for Animal Health, Berkshire, UK

INTRODUCTION

Disease of humans and domestic animals caused by sporozoan blood parasites are a major hindrance to Third World development. Human malaria affects over 300 million people each year and the increasing incidence of drug resistance in *Plasmodium* species has stimulated an intense search for an effective vaccine against the disease. Although much information on the biology and immunology of these parasites has resulted from these efforts, an effective and practical vaccine against human malaria has yet to be developed. *Theileria parva* is a related parasite that causes East Coast fever, an acute lymphoproliferative disease of cattle in eastern, central and southern Africa. Although not as devastating as malaria in human terms, the disease is a huge constraint to the improvement of livestock production in the region and so reduces the capacity of affected countries to feed their expanding populations. This paper will attempt to highlight the common features of *Theileria and Plasmodium* infections and to discuss advances made in the characterization of immune mechanisms that protect recovered cattle against subsequent challenge with *T. parva.*

Because the life cycles of *Theileria and Plasmodium* parasites share a number of features, the approaches followed by scientists in the search for vaccines against these diseases have had much in common. However, a notable difference has been that work on *T. parva* has been conducted entirely in the target host species. Transmitted by *Anopheles* mosquitoes and the *Rhipicephalus appendiculatus* ticks, respectively, sporozoites of both species invade host cells and undergo schizogenous division. After inoculation malaria sporozoites invade a small number of hepatocytes, which rupture after schizogeny and release merozoites. Clinical disease is not apparent until a subsequent expansion of the parasite in erythrocytes. Sporozoites of *T.parva* invade all lineages of bovine lymphocytes, and unlike malaria, their development to schizonts is associated with transformation of the infected cell. By associating with the spindle apparatus, the parasite ensures that each

daughter cell inherits the infection (Fawcett *et al.*, 1984). The extensive invasion of non-lymphoid tissues with parasitized cells that characterizes the disease is associated with this clonal expansion of infected cells. The pathology is also undoubtedly contributed to by cytokine products of the transformed lymphoblasts.

IMMUNE RESPONSES TO *THEILERIA PARVA*

In contrast to malaria, the immune responses to *T. parva* have been characterized extensively in the host species. Much of this information has been derived from animals immunized against the parasite by infection with characterized parasite stabilates in the face of treatment with long acting formulations of oxytetracycline. This method of immunization engenders solid immunity against homologous challenge that lasts at least 31/2 years (Radley *et al.*, 1975; Burridge *et al.*, 1972), but has many disadvantages that preclude its widespread use as a vaccine. These include its dependence on liquid nitrogen for stabilate viability and the possibility of introducing vaccine strains into areas previously free of them. The method has however allowed scientists to establish the basis of the immunity that it induces, as part of a rational approach to the design of an improved *T. parva* vaccine.

It was soon established that cattle immunized by infection and treatment are protected by virtue of cellular mechanisms directed at the schizont-infected cell (Eugui and Emery, 1981; Muhammed *et al.*, 1975; Theiler, 1907). Characterization of these responses was facilitated by the ease with which parasitized cells can be cultured *in vitro*. It was quickly demonstrated that peripheral blood mononuclear cells proliferated in the presence of infected cells and that these cultures contained killing activity (Pearson *et al.*, 1979). This activity in immune cattle was restricted to autologous infected cells, an observation that was reminiscent of cytotoxicity for virus-infected cells in contemporary reports of murine influenza. The MHC-restriction of murine 'flu-specific cytotoxicity prompted an examination of the involvement of bovine MHC products in *T. parva*-specific cytotoxicity. This was made possible by the availability of a large panel of aerological reagents that define bovine class I MHC specificities. In a short time it was established that cytotoxicity in these cattle was parasite-specific and restricted to infected target cells that shared at least one class I MHC specificity with the effector cells (Morrison *et al.*, 1987). In addition, it was shown that killing could be blocked by the addition of monoclonal antibodies specific for monomorphic or appropriate polymorphic determinants on bovine class I MHC antigens. An interesting feature of these experiments was the observation that the restriction of these responses were consistently biased towards one or other haplotype and, further, that certain class I MHC specificities seemed to dominate as restricting elements within populations (Morrison *et al.*, 1987). The subsequent demonstration that parasitespecific cytolytic activity resided in the $CD8^+$ T-cell fraction of peripheral blood confirmed that classical CTL responses are induced in cattle by infection with *T. parva* (Goddeeris *et al.*, 1986).

Several features of CTL responses in *T. parva*-immune cattle implicate this population as major effectors in protective immunity. Their appearance in peripheral blood after challenge is kinetically associated with the onset of patent

parasitosis and its subsequent clearance (Morrison *et al.,* 1987). Studies with two stocks of the parasite have shown that the specificity of the CTL responses in immunized cattle is reflected in the cross-protective properties of the stocks (Morrison *et al.,* 1987; Irvin *et al.,* 1983). More recent studies of this system have indicated that the capacity of an immune animal to resist challenge with a heterologous stock of the parasite appears to depend on the recognition of that stock by its CTL (Taracha *et al.,* 1995). More direct evidence for the role of CTL in immunity to *T. parva* has recently been provided by adoptive transfer experiments carried out in monozygous twin calves (McKeever *et al.,* 1994). Ruminants provide many advantages over human and murine subjects for the study of immune responses *in vivo.* Prominent among these is the ease with which lymphatic vessels entering and leaving lymph nodes can be cannulated for prolonged periods of time with minimal disturbance to the animal. By cannulating the efferent duct of immune lymph nodes under challenge with *T. parva,* it was possible by limiting dilution analysis to determine that parasite-specific CTL constitute as many as 1 in 32 of the cells leaving the node during the peak of the response (McKeever *et al.,* 1994). This frequency can be 25 times that of the same population in concurrent samples of peripheral blood. By eliminating cells of non-$CD8^+$ lineages in responding lymph by complement mediated lysis, over 1 x 10^{10} enriched $CD8^+$ T cells can be prepared from an overnight collection of lymph. These cells can be shown to confer protection on naive twin recipients undergoing lethal challenge with the parasite, and this capacity is abrogated by elimination of $CD8^+$ T cells in the transfer population. These observations provide direct evidence that CTL play a major role in the clearance of *T. parva* from immune cattle under challenge. The induction of these responses is clearly important for the development of an improved vaccine. They also support the belief of many malariologists that CTL against the liver stages of *Plasmodium* parasites might be effective in clearing malaria infections prior to the development of clinical signs.

CONCLUDING REMARKS

The potential of a vaccine against *T. parva* based on a recombinant sporozoite surface antigen is discussed in a separate contribution to this volume. In the light of the observations presented above, and the fact that schizont-specific immunity against *T. parva is* long-lasting, it is clear that an improved vaccine against this parasite should also incorporate a schizont-derived component. The identification of proteins that constitute targets for CTL recognition is a difficult task in any system, and especially so in complex intracellular parasites. It has been estimated that the schizont of *T. parva* expresses approximately 4000 antigens (Gerhards *et al.,* 1989). Based on analyses of the parasite specificities of *T. parva*-specific CTL clones, only a few of these are likely to be CTL targets. Technological advances in the purification and sequencing of peptides associated with cell surface MHC molecules have now provided a means whereby the antigens from which these peptides are derived can be identified (Falk *et al.,* 1991; Hunt *et al.,* 1992). A malaria schizont antigen recognized by CTL in naturally exposed individuals that express the human HLA-B53 class 1 MHC molecule has recently been identified by searching known antigenic sequences of the parasite for a motif derived from peptides isolated from that

molecule (Hill *et al.*, 1992). In addition, it has recently been possible to identify a human CTL target on a melanoma cell line by screening a CDNA library with tumour-specific CTL (Brichard *et al.*, 1993). The necessary materials and reagents for applying these techniques to *T. parva* are available. Large numbers of parasitized cells can be cultured in *vitro* with ease, and the analysis of CTL responses in immune cattle, both at bulk and clonal levels, is considerably less complicated for *T. parva* than for *Plasmodium* species. Accordingly, intense efforts are under way in this institute to apply these technologies to the identification of *T. parva* schizont antigens that constitute targets for protective CTL.

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Class I MHC-restricted immunity in malaria

W. Weiss

Walter Reed Project
Kenya Medical Research Institute
Kisumu, Kenya

The malaria parasite has a complex life cycle involving several stages of development in its mosquito and vertebrate hosts. In the latter, the parasite is intracellular. Following inoculation by a female mosquito taking a blood meal, sporozoite forms of the parasite pass into the liver and within minutes invade hepatocyte cells. Here the sporozoites develop into schizont forms over a period lasting from days to years, depending on the species of host and parasite. The infection is silent during this hepatic incubation: the handful of maturing malaria parasites causes no liver damage and no illness in the host.

When the parasite's development in the liver is complete and it has multiplied many thousand-fold, merozoite parasite forms are released from rupturing hepatocytes into the circulation, where they readily bind to receptor molecules on the surface membrane of erythrocytes. The merozoites enter the red blood cells and, over several days, develop into erythrocytic schizonts. The rupturing of many schizont-infected red blood cells causes clinical malaria. Some of the merozoites, after invading an erythrocyte, rather than multiply further differentiate to sexual forms that can infect mosquitoes. The life cycle is then perpetuated when these sexual forms are ingested by a mosquito taking a blood meal.

The behaviour of the malaria parasite in its mammalian host suggests that this parasite has evolved a survival strategy based on minimizing its exposure to the host's humoral immune responses. These have been demonstrated to be effective against the parasite: both sporozoites and merozoites are rendered non-infective when incubated with antimalarial antibodies (Potocnjak *et al*., 1980; Charoenvit *et al*., 1986).

All mammalian cells except red blood cells express on their surface MHC molecules, which are encoded by genes of the major histocompatibility complex. The expression of surface MHC molecules enables these mammalian cells to present antigen to T lymphocytes of the immune system, which cause the destruction of infected cells. By invading red blood cells, the malaria parasite therefore avoids recognition and attack by MHC-restricted $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T lymphocytes. Other cells of the immune system, such as natural killer cells and gammaldelta T cells, do not require that antigen be presented in association with MHC molecules. Although these cells may recognize parasite antigen on the surface of infected red cells, it is unlikely that $\alpha\beta$ TCR T cells can attack the infected erythrocytes directly. Rather, they may help control infection by interacting with parasite antigens released on the rupture of red cells, by producing systemic cytokines that will induce other protective immune responses, and/or by inducing B-cell responses against the

parasite. MHC-restricted T cells thus play an indirect role in controlling red-cell infection by malaria parasites T-cell responses against the parasite are more direct in the intrahepatic stages of malaria development. Hepatocyte cells express MHC class I molecules and can be induced to express MHC class II molecules. It is thus possible that both $CD4^+$ and $CD8^+$ effector cells recognize and bind to malaria antigens. ($CD4^+$ T cells recognize antigen presented in the binding cleft of class I molecules; $CD8^+$ T cells recognize antigen in the binding cleft of class II molecules.) Under natural conditions, however, few if any people appear to develop strong sterile immunity to the early phases of malaria. This is perhaps because the immune system is exposed to only a handful of sporozoites or developing liver-stage schizont forms in contrast to billions of infected red blood cells.

Much of our knowledge of cellular immunity to the hepatic stages of malaria was gained by infecting human (Clyde *et al*., 1973), rodent (Nussenzweig *et al*., 1976) and avian (Russell and Mohan, 1942) hosts with large numbers of parasites attenuated by radiation. A typical protocol for such immunization involves moderately irradiating infected mosquitoes with X-rays so that the sporozoites remain motile and able to invade their hepatocyte targets. A host is then infected by inoculation through bites from these mosquitoes or through needle injection of sporozoites harvested from the irradiated mosquitoes. Sporozoites irradiated to the point of immobility or administered through intraperitoneal or intradermal routes provoke no protective immune responses in the experimental hosts (Vanderberg *et al*., 1968). The number of sporozoites, as well as boosting doses, needed to immunize a host depends on the species of parasite and host. For example, to protect an individual against disease caused by *Plasmodium falciparum*, the person must be bitten by infected irradiated mosquitoes more than 800 times and over a period of several months (Clyde *et al*., 1975); BALB/c mice, on the other hand, can be protected against disease caused by *Plasmodium berghei* by administration of a thousand irradiated sporozoites in a single inoculation (Jaffe *et al*., 1990).

In this immunization method, the irradiated sporozoites pass into the liver and invade hepatocytes but, because the radiation has damaged their DNA, they are unable to develop into liver-stage schizont forms. Instead, the irradiated sporozoites induce a lymphocytic infiltrate which is absent in hosts infected with healthy sporozoites (Hoffman *et al*., 1989). One effect of the arrested development of the liver-stage parasites is that a lymphocyte inhibitory factor normally made by the hepatic-stage parasites is not produced. Some vaccinologists speculate that irradiated sporozoite vaccines induce T-cell immunity by putting malaria antigens directly into the hepatic cytosol, and thus into the class I, and possibly class II, antigen-processing pathways.

A host immunized with irradiated sporozoites is protected for a period of months to years against disease caused by subsequent infection with up to thousands of healthy sporozoites (Edelman *et al*., 1993). This protection takes two forms. In human and murine malaria, the models most commonly employed, the immunity engendered is one of sterile protection: no blood-stage parasites are detected in immune and protected animals. In breakthrough cases, blood-stage parasites appear and follow their normal patterns of growth without alteration, leading to death or self-cure of the animal host. The second type of immunity induced by irradiated sporozoites, not seen in rodent malaria, was first described in avian malaria (Nussenzweig *et al*., 1967). Young birds immunized

with irradiated sporozoites do develop blood-stage infections after rechallenge with normal parasites, but the severity of the blood-stage infection is much reduced and the birds do not die. This less-studied form of avian immunity may have similarities with natural human immunity to *P. falciparum* malaria in West Africa, discussed below.

The sterile immunity to malaria engendered in mice after immunization with irradiated sporozoites is heavily if not exclusively dependent on effector $CD8^+$, class I-restricted T cells. This has been best illustrated by depleting immunized mice of $CD4^+$ or $CD8^+$ T-cell subpopulations after immunization using monoclonal antibodies (Schofield *et al*., 1987; Weiss *et al*., 1988). Sterile immunity is lost after removing the $CD8^+$ cell population but remains if $CD4^+$ cells are removed. $CD8^+$ and $CD4^+$ T cells that react with malaria antigens can be found in the spleens of immunized animals using *in vitro* assays for proliferation and cytotoxicity. Inbred strains of mice show a genetically restricted immune response after immunization with some but not all murine parasite species (Weiss *et al*., 1989). This response is partly MHC-restricted and partly dependent on genetic factors mapping outside the MHC.

Much less is known about immunity to sporozoite and liver-stage antigens in the human than in the mouse. Irradiated sporozoites of both *P. falciparum* and *P. vivax* induce protective immunity in humans. This immunity is known to be dependent on antigen specificity because no cross-protection occurs (Clyde *et al*., 1973). $CD8^+$ and $CD4^+$ T cells reactive with sporozoite antigens have been identified in the blood of persons immunized with irradiated sporozoites (Malik *et al*., 1991) as well as in people living in areas where malaria is endemic (Doolan *et al*., 1991). However, the relative importance of class I- and class II-restricted T-cell responses has been difficult to assess in humans. Our group in western Kenya is looking for correlations between responses of $CD4^+$ and $CD8^+$ T cells that recognize malaria antigens and resistance to infection by sporozoites.

Work in The Gambia has shed light on the role T cells play in human malaria. A study of human lymphocyte antigen (HLA) types in children with severe malaria (Hill *et al*., 1991) has showed that the HLA Bw53 class I molecule and a HLA class II haplotype were independently associated with less severe disease outcomes during blood-stage infection. This suggests that antigens by both class I and class II MHC molecules are important in protecting the human host. Interestingly, both these HLA types are common in West Africa and very uncommon in other parts of the world, indicating that their protective effects may have fixed them in malaria-exposed populations.

Follow-up work in The Gambia showed that the HLA Bw53 product was able to present a fragment of a *P. falciparum* liver-stage antigen to $CD8^+$ cells (Hill *et al*., 1992). This is a paradox: how can immunity to the liver stages of malaria affect the virulence of infection once the parasite successfully leaves the liver and invades red cells? The question may be answered by looking more closely at avian immunity to malaria induced by irradiated sporozoites: either an immune response in the liver reduces the numbers of infective parasites released into the blood or immune responses in the liver cross-react with blood stages of human malaria.

IMMUNIZATION STRATEGIES IN MALARIA

With the understanding of the importance of class I MHC-restricted T-cell immunity in malaria, a variety of techniques have been used in attempts to induce immunity to sporozoite and liver-stage antigens.

Irradiated Sporozoite Vaccines

Until recently, malaria sporozoites could be grown only in live mosquitoes. Producing sporozoites for mass immunization was thus highly impractical. Recent breakthroughs in growing sporozoites *in vitro* (Warburg and Miller, 1992) may solve the problem of mass sporozoite production. However, immunization with irradiated sporozoites will probably continue to be constrained by the necessity for the immunizing sporozoites to remain able to invade liver cells.

Recombinant Live Vector Vaccines

One way to introduce malaria antigens into the class I MHC pathway is to incorporate the genes encoding these antigens in the genetic material of other intracellular organisms. Researchers have successfully expressed malaria parasite antigens in vaccinia viruses (Sedegah *et al*., 1988) but no such recombinant virus has yet been able to induce in the host protective immune responses or cytotoxic T lymphocytes (CTLS) that specifically recognize the antigens. Use of recombinant attenuated bacteria to deliver antigen has shown greater promise. *Salmonella* strains with an *Aro* A mutation are able to invade cells but are then unable to multiply. In an encouraging experiment with mice, however, the addition of a plasmid expressing the malaria circumsporozoite antigen to a non-virulent *Salmonella* bacterium protected 70 per cent of the immunized mice against a subsequent sporozoite challenge (Sadoff *et al*., 1988). Not all vaccinations with recombinant *Salmonella* have protected mice against malaria, however, despite the fact that the vaccinations induced CTL responses (Flynn *et al*., 1990). Research in this area continues in attempts to make a comparable, protective *P. falciparum* vaccine for humans.

Synthetic Peptide Vaccines to Induce Class I MHC-Restricted Immune Responses

Attempts are being made to use chemically synthesized peptides corresponding to fragments of malaria parasite antigens as the bases for malaria vaccines. The aim of most of these attempts is to induce responses from $CD8^+$ T cells. along with antibody and class II MHC-restricted immune responses.

Unmodified proteins and short peptides can induce CTLs when given in very high doses with incomplete Freund's adjuvant (Carbone and Bevan, 1989). These techniques have been tried in the mouse malaria models, using peptides representing a CTL epitope from the circumsporozoite protein. Significant $CD8^+$ T-cell responses have been induced, but these peptide vaccines have not protected mice against parasite challenge (W. Weiss, unpublished observations).

Modified peptides can also induce $CD8^+$ T-cell responses and often require much lower quantities of antigen than unmodified peptides. Adding covalently coupled lipids to peptides reduces one hundred-fold the amount of peptide required to induce CTL; this has been demonstrated for the malaria circumsporozoite antigen in the mouse (Verheul *et al.* 1993). Such modified peptides can be further combined into particle forms, such as ISCOMs (Takahashi *et al.*, 1990) or liposomes. For example (White *et al.*, 1993), a modified circumsporozoite protein from the human *P. falciparum* parasite with a repeating motif deleted was incorporated into liposomes with the adjuvant monophosphoryl lipid A. A single 10-microgram intraperitoneal injection induced CTLS. However, such modified peptide and protein vaccines have not yet successfully protected mice against malaria sporozoite infection.

Induction of Class I MHC-Restricted Responses by Injection with DNA

An extremely novel technique for inducing class I MHC-restricted immune responses has recently been described for viral antigens (Ulmer *et al.*, 1993) and is also showing great promise for malaria (M. Sedegah and S.L. Hoffman, manuscript in preparation). Plasmids containing complementary DNA (cDNA) sequences from influenza nucleoprotein and malaria circumsporozoite antigens were injected intramuscularly into mice. The plasmids were taken up intact by muscle cells, where they resided without multiplying. The cDNA sequences were transcribed to make intracellular proteins corresponding to the nucleoprotein or circumsporozoite antigens. Although the proteins were produced at extremely low levels, significant antibody, $CD4^+$ and $CD8^+$ T-cell responses are made to these antigens. Most excitingly, the immunized mice in some of these experiments were protected against influenza and malaria.

Problems with Class I MHC-Restricted T Cells and Protective Immunization

Both $CD8^+$ (Romero *et al*, 1989; Weiss *et al.*, 1992) and $CD4^+$ (Tsuji *et al*, 1990) mouse T-cell clones that recognize particular malaria parasite epitopes have been shown in passive transfer experiments to protect animals from malaria. However, when animals are immunized with constructs that induce $CD8^+$ responses to these same epitopes, there is no evidence that the parasite is

affected. Ibis raises the possibility of the induction of protective immune responses is brought about not only by the activity of CTLs but also by special characteristics of those CTLS.

One such characteristic is the presence of the correct adhesion molecules on the surface of the CTL. A study of protective and non-protective CTL clones (Rodgigues *et al,* 1992) showed that the presence of CD44 and VLA on the surface of these cells correlated with an ability to protect mice in passive transfer experiments. Non-protective clones did not home to their targetinfected hepatocytes—as well as protective clones. The reason so many immunization strategies that induce CTL proliferation do not protect the host against malaria infection may be that the induction does not produce class I MHC-restricted T cells with the necessary homing characteristics.

We have recently published experimental results showing that $CD4^+$ cell activity is required for development of protective $CD8^+$ cells in mice immunized with irradiated sporozoites (Weiss *et al.*, 1993). Mice depleted of $CD4^+$ cells at the time of immunization developed the same numbers of $CD8^+$ cells in their spleens and livers as did the controls but were completely unprotected against malaria sporozoites. Although we were not able to define the critical action $CD4^+$ cells provide in a protective immune response, it may be related to the development of a special characteristic of $CD8^+$ effector cells.

Interactions *in vivo* among T-cell subpopulations are almost certainly crucial to the success of a vaccine against malaria. It is possible that many of the vaccine constructs mentioned here, which successfully induce $CD8^+$ responses but do not protect against the parasite is that the constructs are not inducing properly coordinated $CD4^+$ and $CD8^+$ T-cell responses. The rational design of a synthetic malaria vaccine may therefore require further study of the induction phase of CTLs in animal models and in humans.

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The selection of class I MHC-restricted epitopes

T. Elliott

Molecular Immunology Group
Institute for Molecular Medicine
John Radcfiffe Hospital
Oxford OX3 9DU, UK

INTRODUCTION

Cellular immunity mediated by cytotoxic T.cells (CTL) is an essential part of the specific immune specific response to viruses and other intracellular parasites. CTL recognize a complex ligand at the surface of infected cells comprising pathogen-derived peptide epitopes bound to glycoproteins encoded by the class I locus of the major histocompatibility complex (class I MHC). The study of class I MHC at the micromolecular level, and of the peptides that are naturally bound by them, has provided a detailed picture of the complex recognized by CTL.

How class I MHC molecules select peptides from a potentially large intracellular pool of pathogen-encoded proteins is as yet unclear, and an understanding of these selection processes is crucial to the assessment of vaccine strategies designed to prime the class I MHC-restricted arm of the immune system. Selection could operate at the level of proteolysis, translocation of peptides from the site of production to the site of their interaction with MHC, or the MHC binding event itself. This paper briefly reviews what is known about each of these events with respect to their permissiveness.

COMPLEMENTARITY BETWEEN CLASS I MHC AND BOUND EPTIDES

By isolating class I MHC molecules and stripping them of bound peptides by denaturation, it has been possible to purify a representative, heterogeneous mixture of ligands bound to a number of human and murine allele products. Sequencing of this mixture has revealed that all bound peptides are homogeneous in length, and share common amino acids at particular positions in the sequence—described as anchor residues (Falk *et al.,* 1991). Because class I MHC associated peptides are homogeneous in length, the positions of these anchor residues within the sequence thus defined allele-specific binding motifs. Examples of such motifs are shown in Figure 1.

The molecular basis of complementarity between class I MHC and its bound peptides was elucidated by high resolution X-ray crystallography and confirmed by biochemical approaches. The two membrane-distal domains of class I MHC

Figure 1. Peptide binding motifs for several class I MHC alleles in mouse and man.

MHC	Peptide residue						
	1	2	3	4	5	6	7
H2–Kb					F y		L
H"–Db					*N*		M I
H2–Kd I		y					L
H2–Kk		E D					
H2-Ld		P					L
HLA-A2.1		L					v L
HLA-B35		P					y
HLA-B53		P					F W
HLA-B8			K		K		I
HLA-B27		R					K R
HLA-B7	A	p	R				L v
HLA68.1		T V					R
HLA-A3.1		I L	F				K Y
HLA-All		I L					K

heavy chain form a binding groove lined with many of the polymorphic residues and contoured in such a way as to define six pockets (Sapar *et al,* 1991). Crystallographic evidence has shown that the function of four of these pockets is to accommodate side chains of anchor residues within the bound peptide. Hence the arginine side chain found at position 2 of all HLA-B27-binding peptides fits into a deep 'B' pocket at the end of which is found a glutamic acid residue(Maden, *et al,* 1992). In some alleles, this pocket is obscured by a bulky side chain at either position 67 or 45 with the result that either other pockets are utilized, or peptides with proline at position 2 are preferred. The pocket that defines the 'left hand' end of the groove is lined with non-polymorphic hydrogen bond donors such as tyrosine, and interacts with the alpha amino group of bound peptides. The pocket defining the 'right hand' end is similar and makes a series of hydrogen bonds between conserved residues and the free carboxy terminus of bound peptides. In addition, a correlation has been noted between the nature of the side-chain at position 116 (which forms the floor of this pocket), and the

side chain of the C-terminus residue of the bound peptide (Elliott *et al.*, manuscript in preparation). Thus, aromatic side chains are found in this position in those alleles that prefer a C-terminal hydrophobic residue, serine is found in alleles that prefer aromatic C-termini, and aspartic acid is always found at position 116 of alleles that bind peptides with basic C-termini.

CLASS I MHC ASSEMBLY

Peptide binding and class I MHC assembly are linked phenomena. A study of the assembly of class I with peptides *in vitro* has shown that peptide binding of the 'empty' heavy chain/beta 2-microglobulin heterodimer stabilizes the interaction between the two class I MHC chains. Furthermore, peptides binding to free heavy chain in the absence of beta 2-microglobulin has been shown to induce a conformational change in heavy chain leading to its native structure-in which it binds to beta 2-microglobulin with high affinity (Elliott *et al.,* 1991). Either of these reactions could contribute to the selection of intracellular epitopes since 1) Peptides of 9 amino acids bind with the highest affinity to 'empty' class I molecules (Cerundolo *et al.,* 1991) and 2) only peptides corresponding to naturally processed epitopes will induce the conformational change in free heavy chains (Elliott *et al.,* 1991). The last of these may be particularly important, since the alpha-3 domain of heavy chain is not required to observe the effect, and there is some evidence to suggest that peptides of an optimal length and sequence may actually lower the activation energy required for correct folding of the free HC in the absence of beta 2-microglobulin (Elliott *et al.*, 1992a, 1992b).

PEPTIDE TRANSPORT

Current evidence supports the notion that the primary site of pathogen protein degradation is the cytosol. Class I MHC is synthesized and binds to peptides in the lumen of rough endoplasmic reticulum (ER), and it was clear from an early stage that the topological paradox which these observations pose could only be resolved if there exists a mechanism for translocating peptides across the ER membrane in a signal sequence-independent way. Recently, a heterodimeric protein encoded in the MHC class II locus has been identified that fulfils this role. The complex, known as TAP.(Transporter associated with Antigen Processing, reviewed in Townsend and Trowsdale, 1993), belongs to a family of ATP-binding cassette (ABC) proteins and has been shown to transport peptides from the cytosolic to the lumenal side of the ER membrane in an ATP-dependent way (Neefjes *et al.,* 1993; Shepherd *et al.,* 1993). The two TAP chains are genetically polymorphic and there is one instance in which this has been associated with functional polymorphism (Powis *et al.*, 1992). Differences in substrate specificity of TAP alleles could therefore contribute to the selection of cytosolic peptides that could be made available for class I MHC binding in the ER. Studies to date clearly show that the TAP complex is not selective for nonameric peptides, but can transport peptides up to 15 amino acids in length.

PROTEOLYSIS

The best-studied agent of protein degradation in the cytosol is a multicatalytic complex known as the proteasome. Two of the proteasome subunits are polymorphic gamma-interferon inducible and encoded by the class II locus of MHC (Martinez and Monaco, 1991). Recent evidence shows that of the three ATP-independent proteolytic activities of the proteasome, those which cleave after hydrophobic and basic amino acids are augmented after interferon gamma treatment, whereas the third, which cleaves after acidic residues is suppressed (Driscoll *et al.,* 1993; Gaczynska *et al.,* 1993). The modulation of these activities appears to be dependent on the presence of the two MHC-encoded subunits. Thus, under conditions found during immune activation, the proteasome may preferentially generate peptides with C-termini that are compatible with all class I MHC alleles studies to date, while reducing the generation of peptides with acidic C-termini (for which a complementary class I MHC binding groove has not been identified). Down regulation of the acidic specificity would also prevent peptide epitopes containing acidic residues from being destroyed.

The same abundance of basic and hydrophobic residues seen at the C-termini of peptide epitopes is not evident at the position immediately preceding the N-termini. Here, the relative abundance of each amino acid approximates to the frequency with which they occur in nature, suggesting that different sets of enzymes may be involved in generating the N-termini of peptide epitopes. We have recently investigated the role of enzymes resident in the ER in trimming longer peptides containing a natural epitope (Elliott *et al.,* 1993). Long precursor peptides generated by recombinant vaccinia viruses were co-translationally transported into the ER of a mutant cell line that is unable to expose newly synthesized class I molecules to peptides generated in the cytosol. Although the longer peptides were shown to bind to class I MHC with a fairly high affinity, only the 'naturally processed' nonameric peptide could be isolated from class I MHC molecules synthesized by the infected cells. These results indicate that longer peptides transported into the ER (either by a TAP-dependent mechanism, or by leader sequence-dependent mechanisms as in this experiment) could be trimmed by ER-resident peptidases giving rise to peptides capable of inducing tiie folding of nascent class I MHC and forming stable complexes with them.

CONCLUSIONS

An effective vaccine should incorporate all the elements necessary for its processing and presentation to appropriate precursor T-cell subset(s). In general, vaccination strategies targeted at MHC class I-restricted responses fall within a spectrum ranging from infection/treatment to immunization with defined epitopes. Both of these extremes are suboptimal. Although infection with a pathogenic organism is likely to prime appropriate effector and regulatory T.cells, there are obvious disadvantages to using live organisms and the strategy is limiting to those diseases for which a suitable and reliable treatment is available. On the other hand, peptide vaccination strategies limit the response to a single epitope/class I immunostimulatory complex which may

be ineffective or even detrimental if the vaccinating epitope differs in any way between individuals (of the same haplotype) or is not produced throughout the course of the pathogenic challenge. In addition, there may be as yet undefined disadvantages associated with delivering a bolus of preprocessed antigen to the circulation.

In the absence of detailed information regarding the processing requirements of intracellular antigens for presentation by class I MHC, vaccination strategies have tended to focus on attenuated live vaccines designed to deliver a number of potentially immunogenic proteins to intracellular processing components in a non-pathogenic form. Though these vaccines may be effective in delivering antigens in a way analogous to that of the wild-type pathogens, there are many difficulties associated with the approach, not least of which is the worry over effective attenuation. Also, the approach is only feasible in cases where the pathogen is simple (i.e. viruses with a small genome) and its biology is well understood.

What remains, therefore, are strategies that draw on information derived from the study of antigen processing and presentation. Since this information is currently limited, the popular strategies focus on the delivery of immunodominant antigens to the cytosol of antigen presenting cells by, for example, enclosing them in liposomes or making them fusogenic by some other means. Clearly, a more detailed knowledge of the way in which antigens are handled by antigen presenting cells prior to their association with class I MHC, and an understanding of the structure of the class I molecules which present them, will enhance our ability to approach the development of effective vaccines in a rational way.

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Search for CTL-recognized peptide epitopes

H.J. Stauss, E. Sadovinikova, L. Yin, P.Nathan and C. Thomas

Imperial Cancer Research Fund
Tumour Immunology Unit
91 Riding House Street
London WC I 8BT, UK

Cytotoxic T.lymphoeytes (CTL) recognize peptide fragments presented on the cell surface by MHC class I molecules. Peptides are generated by proteases in the cytosol and transported across the membrane of the endoplasmic reticulum where they bind to newly synthesized MHC molecules. However, not only cytosolic proteins can give raise to MHC presented peptides, since nuclear (Deckhut *et al.,* 1992) and even mitochondrial (Loveland *et al.,* 1990) proteins have been shown to provide MHC class I-presented CTL epitopes. At present, no cellular compartment is known to be excluded from the MHC class I presentation pathway. Indeed, CTL responses have been detected against bacteria and parasites that reside inside cellular vacuoles (Kaufmann, 1993) and are therefore probably not exposed to cytosolic antigen processing enzymes. It is currently unknown whether generation of immunogenic peptides from such intravacuolar parasites is dependent on cytosolic proteases and peptide transporters, or whether alternative antigen processing pathways exist.

MHC class I binding is an essential prerequisite for peptides to function as CTL epitopes. Sequence analysis of MHC-bound peptides in combination with crystallographic structures of MHC class I molecules led to the definition of rules governing peptide-MHC interaction The peptide binding groove of class I molecules is highly polymorphic, and this results in binding of distinct sets of peptides by different MHC alleles. Study of peptide-MHC interaction resulted in determination of allele-specific peptide binding motifs (Falk *et al.,* 1991), which have been used successfully for accurate prediction of many CTL epitopes (Falk and Rotzschke, 1993). However, CTL epitope prediction based on these observations requires protein sequence information of the target antigen, which is often not available. Also, not all natural CTL epitopes contain MHC binding motifs (Sadovnikova *et al.,* 1994) and the frequency of motif-negative epitopes is presently unknown.

IDENTIFICATION OF CTL EPITOPES

Approaches for identification of CTL epitopes without knowledge of target proteins have been described. Boon's group developed a procedure that involves generation of variant cells that have lost expression of CTL-recognized epitopes, followed by transfection of these variant cells with genomic DNA from epitope-positive parental cells. This approach has led to the identification of tumour antigens expressed by human melanoma (Traversari *et al.,* 1992) and

murine mastocytoma cells (Sibile *et al.*, 1990). The drawback of this technique is that it is extremely labour intensive, and to date it has been used successfully in only one laboratory.

An alternative approach to identify CTL epitopes involves isolation and purification of MHC class I-presented peptides followed by peptide microsequencing. However, MHC class I-presented peptides are highly heterogeneous (estimates are that probably 3,000 to 10,000 distinct peptides are presented per cell) requiring extensive purification before sequence of individual CTL-recognized peptides can be obtained. The most serious limitation of this approach is the discrepancy in sensitivity of CTL assays and peptide microsequencing. The sensitivity of classical Edman degradation sequencing is 1-10 picomole and the sensitivity of current tandem mass spectroscopy is approximately 50 femtomole. Estimated CTL sensitivity is 1–10 femtomole, indicating that successful purification of CTL-recognized peptides does not guarantee successful sequence determination. Nonetheless, four CTL epitopes have been microsequenced successfully in three different laboratories to date (Udaka *et al.*, 1992) (unpublished information). Surprisingly, in most cases classical Edman degradation was employed for sequence determination, while the technically more advanced tandem mass spectroscopy was used for identification of one epitope recognized by allo-reactive CTL specific for HLA-A2.

In our own work we have analysed peptides recognized by CTL raised against minor histocompatibility (H) antigens. Minor H antigens can cause tissue transplant rejection in MHC-matched, donor-recipient combinations, due to CTL recognition of polymorphic non-MHC antigens. Except for two characterized minor H antigens in mouse (Loveland *et al.*, 1990; Speiser *et al.*, 1990), the nature of these antigens and the encoding genes are generally unknown. We have studied the murine minor Hl antigen which is recognized by CTL in a MHC class I, H-2Kb-restricted fashion (Yin *et al.*, 1993). So far, we have immunopurified Kb molecules from approximately 30 mouse spleens and separated Kb-presented peptides by reverse phase HPLC. Individual HPLC fractions were collected and tested in CTL assays showing that only one or two adjacent HPLC fractions contained peptides recognized by CTL specific for the minor HI antigen (Yin *et al.*, 1993). Active fractions were subfractionated over three different HPLC columns and CTL-recognized peptides were successfully identified. Subfractionation revealed that the original HPLC fractions contained a large number of irrelevant peptides that interfere with microsequencing of CTL-recognized peptides. After the final HPLC purification the CTL-recognized fractions contained approximately 1 picomole of peptide which was sufficient to obtain some sequence information using Edman degradation. These purification experiments were encouraging because biologically active peptides were easily identified even after four purification steps. Our current effort is to use more efficient peptide isolation and separation procedures for purification of CTL epitopes.

ISOLATION OF SURFACE PEPTIDES FROM INTACT CELLS

We have developed a peptide extraction protocol which does not require prior immunopurification of MHC class I molecules. This procedure is likely to improve peptide yield because it omits potential losses associated with

immunopurification. Also, the procedure is independent of the availability of MHC-specific antibodies, allowing peptide isolation from many class I alleles of different species. Denaturation of MHC class I molecules on the surface of intact cells is used to release MHC-bound peptides, which are subsequently purified by HPLC. To monitor MHC class I denaturation, cells were exposed to buffers of pH 6.5, 5.6, 4.5 or 2.7 and then stained with conformation-dependent, MHC class I-specific monoclonal antibodies. Exposure of cells to pH 6.5 or 5.6 did not significantly reduce levels of class I expression, while exposure to pH 4.5 lead to a slight reduction (Figure 1). MHC class I expression was reduced to undetectable levels in cells exposed to pH 2.7. Exclusion of trypan blue dye by these cells indicated that pH 2.7 exposure did not damage the integrity of the cell membrane. Therefore, it appeared that pH 2.7 resulted in denaturation of class I molecules without causing detectable cell damage. Ph 6.5 and 5.6 did not affect MHC expression and pH 4.5 appeared to induce partial denaturation of class I molecules.

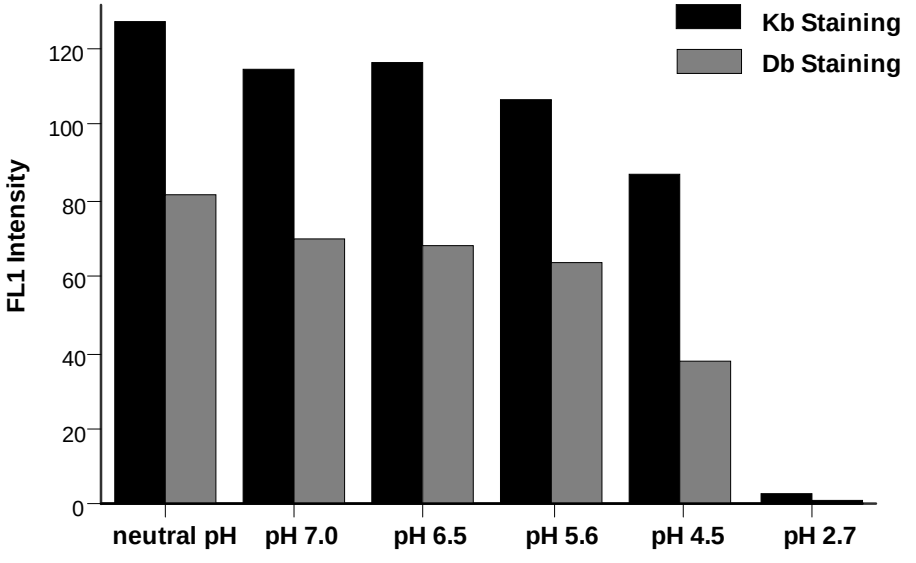

Figure 1. Denaturation of MHC class I molecules on the surface of intact cells. RMA cells (H-2b haplotye) were exposed to pH-adjusted buffers (500 mM NACI and 200 mM acetic acid) for 3.5 min at ro(,m temperature, followed by staining at 4 ?C with murine MAbs specific for H-2Kb and Db class I molecules and second layer FITC-labelled goat anti-mouse antibodies. Shown is the median of the fluorescence intensity of the stained cells as determined by FACScan analysis. The morphology of cells exposed to low pH was similar to that of control cells, and pH-treated cells did not show signs of cell damage when analysed by trypan blue staining.

Supernatant of cells exposed to the pH-adjusted buffers was collected and tested for the presence of peptides recognized by CTL raised against the minor H l antigen. Supernatants of cells exposed to pH 6.5 and 5.6 did not contain any CTL-recognized peptides, while supernatants from cells treated with pH 2.7 were efficiently recognized by CTL (Figure 2). Treatment with pH 4.5 appeared

to lead to partial release of CTL-recognized peptides (Figure 2). Together, these experiments revealed a good correlation between the disappearance of MHC class I molecules on the surface of pH treated cells, and the appearance of peptides recognized by class I restricted CTL (see Figures 1 and 2). Peptide release from intact cells appeared to be efficient, since titration experiments suggested that the concentration of pH eluted peptides is comparable to the amount of peptide obtained from lysates of whole cells (data not shown).

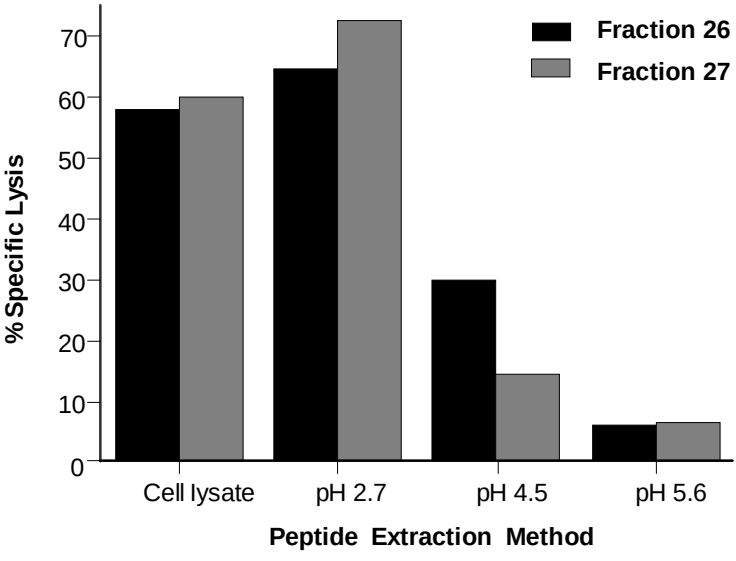

Figure 2. CTL recognition of natural peptides eluted from intact cells. 2.5 x 10^8 RMA cells were exposed to pH-adjusted buffers for 3.5 min at room temperature. Cells were removed by centrifugation and the supernatant passed through Centricon 10 filters to isolate low molecular weight peptides which were separated by HPLC. Fractions 26 and 27 were collected and analysed with CTL specific for the minor histocompatibility antigen HI, which recognize peptides present in these HPLC fractions (Yin *et al.*, 1993). Peptides prepared from cells by lysis with 0.7% TFA served as positive control. For CTL assays, 1/10 of HPLC fractions 26 and 27 were used to coat RMA-S target cells (uncoated RMA-S cells showed approximately 5% background lysis).

Although pH 5.6 exposure did not result in MHC denaturation and release of CTL-recognized peptides, HPLC analysis of pH 5.6 eluent revealed the presence of several peptide peaks (not shown). Although the origin of these peptides has not been determined, it is possible that they are released from receptor ligand interactions which are more ph-sensitive than MHC/peptide interaction. For example, it has been shown that pH 5.6 can cause dissociation of epidermal growth factor bound to its receptor (Haigler *et al.*, 1980). Since omission of such peptides should improve the purity of CTL-recognized peptides, cells were pre-exposed to pH 5.6 to elute irrelevant peptides before MHC presented peptides were extracted using pH 2.7. The pretreatment at pH 5.6 did not result

in any detectable loss of CTL-recognized peptides and was therefore routinely used for peptide preparation.

The experiments described here were designed to develop procedures for rapid peptide extraction from a large number of MHC class I molecules. A major advantage of the described surface extraction procedure is the independence of MHC-specific antibodies allowing peptide extraction from class I molecules for which no antibodies are available. However, peptides prepared in this way are more heterogeneous than peptides extracted from immunopurified class I molecules. This is because surface extraction leads to denaturation of all MHC molecules expressed by treated cells, and the heterogeneity of released peptides increases with the number of expressed class I alleles. This drawback of increased heterogeneity is perhaps compensated by improved peptide yield and by the experimental facility of peptide preparation.

CONCLUDING REMARKS

It is currently unknown whether peptide purification and microsequencing can be achieved in the majority of CTL-recognized epitopes or in only a few exceptional cases. An important factor determining microsequencing success is probably the level at which a CTL epitope is expressed, which in turn determines the concentration of purified peptides. At present there is only some information of the minimal number of MHC/peptide complexes that are required for CTL recognition, but no assays are available to measure the actual concentration of a given MHC/peptide complex. In addition to expression level the biochemical nature of peptides is likely to affect purification and microsequencing attempts. To date, most peptide isolation procedures use low pH buffers which may result in loss of some peptides that are sensitive to acidic conditions. This may explain our observation that acid extraction does not always yield CTL-recognized peptides. For example, we have generated several H-2Kb-specific CTL clones which recognize RMA cells but not the mutant RMA-S subline which has an MHC class I peptide loading defect. Although acid extraction of parental RMA cells yields peptides recognized by most CTL clones, some clones apparently recognize epitopes that are not present in these peptide preparations. It is possible that peptides recognized by these clones are unstable under low pH conditions. Alternatively, it is conceivable that HPLC purification may not give equally good recovery of all peptides, leading to loss of some CTL epitopes. Finally, different CTL may display different affinities for peptide MHC complexes in which case lack of CTL recognition might be caused by low affinity T-cell receptors rather than by low peptide concentration.

Attempts to microsequence CTL epitopes are currently undertaken in many different laboratories and within the next few years it will be possible to judge the general feasibility of this approach.

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Population diversity and natural selection of class I HLA molecules

P. Parham

Departments of Cell Biology and Microbiology and Immunology
Sherman Fairchild Building, Stanford University
Stanford, California, 94305–5400, USA

INTRODUCTION

The HLA region lies on the short arm of human chromosome 6 and contains approximately 20 class I MHC heavy chain genes, pseudogenes and gene fragments (Geraghty *et al.*, 1992). Three genes encode serologically detected HLA-A, B and C antigens, which are expressed by a wide range of tissues and function in the presentation of peptide antigens to $CD8^+$ T.lymphocytes.

Serological typing, using alloantisera obtained from multiparous women, has been the primary tool for characterization of HLA-A, B, C polymorphism in human populations and remains the routine method for clinical Class I MHC typing. With this approach, the activities of eleven international histocompatibility workshops have defined 27 HLA-A, 59 HLA-B and 10 HLA-C antigenic specificities. Because some of these specificities represent shared public epitopes the number of serotypically defined alleles are 23 for HLA-A, 46 for HLA-B and 9 for HLA-C.

Historically, HLA studies have been carried out in the clinical context of tissue transplantation and autoimmune disease and have therefore focused upon Europeans and derivative populations. The major source of anti HLA alloantisera and of the cells used to screen them have been individuals from these same populations. In 1972 the fourth International Histocompatibility Workshop focused on the diversity of human populations. New antigens specific to non-European populations were discovered and striking differences in antigen frequencies between populations revealed. For example, the numbers of HLA-A and B antigens found in Native American populations were only fractions of those found in Non-Native Americans (Kostyu and Amos, 1981).

Not long after the 1972 workshop the role of class I HLA molecules in antigen recognition by T.lymphocytes began to be appreciated, stimulating study of the HLA specificity of alloreactive and antigen-specific T.cells. Unexpectedly, T.cells were found to distinguish between serologically identical class I MHC molecules. This was first recognized with HLA-A2, and then with A3, B27, B35, B44 and others (Biddison *et al.*, 1980). Primary sequence analysis showed that the basis for these differences were amino acid substitutions in the heavy chain sequence. In this manner the HLA-A2 and HLA-B27 specificities were shown to represent a collection of related alleles, which have been called subtypes (López de Castro, 1989). As more serologically defined antigens have been analyzed using T.cells, isoelectric focusing and nucleotide sequencing, a

substantial number have now been dissected into subtypes. A common feature of subtypes is that they segregate between populations. Hence different, HLA-B27 subtypes derived from Africa, India, eastern Asia and Europe have been found (Benjamin and Parham, 1990).

CORRELATING SEQUENCES AND SEROLOGY

Nucleotide sequences for all the WHO-recognized HLA-A, B and C antigens have now been obtained (Zemmour and Parham, 1993; unpublished data). These data enable correlation of the primary sequences with the patterns of aerological cross-reactivity previously defined.

HLA-A

HLA-A locus antigens have been divided by aerological analysis into five cross-reacting antigens (CREG) designated A2, A3, A9, A10 and A19 (Rodey and Fuller, 1987). On the basis of nucleotide sequences five families of alleles can be defined (Kato *et al.*, 1989; Lawlor *et al.*, 1991) and these correspond to the five CREG. The exception to this rule are the A30 subtypes, which serologically belong to the A19 group but show sequence characteristics of the A3 group. The structural similarities that create the aerological epitopes sharedby A30 and the A19 family (A29, A30, A31, A32 and A33) are located between residues 144–151 of the a_2 helix. HLA-A30 molecules are more frequent in Africans and African Americans than in Europeans, which perhaps explains why serology has focused on such a limited region of the A30 molecule. If alloantisera obtained from individuals stimulated with a cross-reactive antigen were used to define the A30 antigen then serology would focus only on the cross-reactive determinants.

Potential problems associated with defining antigens from one population with alloantisera characterized in another are illustrated by an unusual and recently discovered allele, HLA-A*8001. In the African American population 6.7% of HLA-A alleles type serologically negative or 'blank'. Sequencing of such blank alleles has revealed the A*8001 allele, which was discovered independently by three groups of investigators (Domena *et al.*, 1993; Starling *et al.*, 1993; Rosen-Bronson *et al.*, 1992). HLA-A*8001 is very different from other HLA-A alleles and on the basis of nucleotide sequence comparisons defines a sixth allelic family. Its closest relative is A*0101 from which it differs by 34 nucleotide and 24 amino acid substitutions. By superimposing the A* 8001 amino acid sequence on the three dimensional structure of the class I molecule (Bjorkman *et al.*, 1987a, 1987b), it can be seen that substitutions distinguishing A*0101 and A*8001 are exposed on the surface of the molecule and thus expected to be antigenic. Given the distinctive nature of the A*8001 molecule it is remarkable that it escaped aerological detection for so long. This does not appear to have been due to poor immunogenicity of the A*8001 molecule, because recent screening of the appropriate population has revealed A*8001 specific alloantisera (Starling *et al.*, 1993).

Serological definition of HLA-B CREG has been less clear than that of HLA-A and a similar complexity is seen in the HLA-B nucleotide sequences, which do not easily subdivide into families like HLA-A alleles. Variation is heavily concentrated in exons 2 and 3 and the evolutionary lineages have become obscured through gene conversion events which have resulted in recombination of short sequence motifs within the region (Parham *et al.,* 1988).

Comparison of HLA-A and B with the chimpanzee homologues shows that the A alleles are more closely related than the B alleles (Lawlor *et al.,* 1988; Mayr *et al.,* 1988). This observation, indicates that the B locus has evolved more rapidly than the A locus during the five million years since human and chimpanzees diverged, and this is supported by the comparison'of HLA-A and B within the human species (Belich *et al.,* 1992; Watkins *et al*., 1992). Moreover, the primary difference between the two loci appears to be the frequency with which new alleles formed by interallelic gene conversions enter the gene pool.

The degree to which aerological cross-reactivity between HLA-B molecules reflects overall structural similarity is highly variable. The Bw4 and Bw6 public epitopes, formed by alternative sequence motifs at residues 77–83 of the al helix (Wan *et al.,* 1986) are influential in determining the aerological status of HLA-B molecules. A series of crossreactive antigenic pairs has been identified (B8/B59, B44/B45, B49/B50 B38/B39, B35/B53, B51/B78 and B62/B63) in which one member of the pair has the Bw4 epitope and the other the Bw6 epitope. In the absence of structural information it was speculated that these pairs of antigens would differ solely in the sequences determining the public epitopes. With determination of the sequences it became apparent that the pairs divided into two groups, those that fulfilled this expectation and those that did not.

Four of the pairs (B49/B50, B38/B39, B35/B53 and B51/B78) differed as predicted, in the motifs at positions 77–83 (Müller *et al.,* 1989; Hildebrand *et al.,* 1992; Hayashi *et al.,* 1990; Sekimata *et al.,* 1990; Madrigal *et al.,* 1992). For the other three pairs (B8/B59, B44/B45 and B62/B63) many additional differences were found and it was not obvious from the structures why the alleles were serologically paired (Hildebrand *et al.,* 1992, 1993, 1994). On the basis of sequence B59 is more closely related to B55 than B8 (Hildebrand *et al.,* 1993), B45 is more closely related to B50 than B44 (Hildebrand *et al.,* 1992) and B63 is more closely related to B 17 than B62 (Hildebrand *et al.,* 1994). The lack of congruity between serology and structure results from dominant epitopes formed by highly localized segments of sequence and from the antigenic specificities being defined on the basis of cross-reactivities: B59 is specific to Japanese populations and B45 and B63 are most common in Africans and derivative populations (Imanishi *et al.,* 1992).

Another example of this effect emerged from analysis of HLA in the Piman Indians. In this population a aerological variant of the HLA-B21 antigen, designated BN21, was defined (Williams and McAuley, 1992). This was intriguing because neither of the conventional B21 antigens, B49 and B50, were found in any American Indian groups (Kostyu and Amos, 1981) and BN21 was restricted to the Pima and certain closely related Native American populations. The sequence of the BN21 allele proved to be highly divergent from B49 (B*4901) and B50 (B*5001), but was very similar to B*4002, an allele that is

common among American Indian groups, including the Pima, and encodes the B61 antigen (Hildebrand *et al.*, 1992; Domena *et al.*, 1992). The BN21 molecule differs from B *4002 by substitution at positions 152 (V « E) and 163 (T.« L) in the a2 helix. At these two positions the BN21 allele, now called B*4005, is identical to B*4901 and B*5001, which must explain the aerological cross-reactivity. This sequence is present in other alleles including ones found in the P»ma, for example B*5101 and B*5102. Therefore, the B*4005 allele was formed by a conversion in which B*4002 was the acceptor and either B*5101 or B*5102 was the donor. In conclusion, a B21-like antigen was formed by recombination between two alleles, neither of which was in the B21 family: a sort of convergent evolution.

HLA-C

Serological definition of HLA-C has proved difficult and there have been few developments in this area in the last decade. Similarly, the characterization of HLA-C proteins has been hindered by the lack of either allele-specific or locus-specific monoclonal antibodies. The most successful of the analytical approaches applied to HLA-C have been those directed at nucleic acids. Analysis of HLA-C allelic sequences reveals intriguing differences from HLA-A and B: the peptide binding site is less polymorphic, especially in the al helix, yet there is greater variation at positions outside the peptide binding site (Zemmour and Parham, 1992). Many research groups are actively engaged in the analysis of HLA-C and the emerging impression is that there is much undiscovered polymorphism at this locus.

DNA Methods for HLA-A, B, C Typing

Until recently serology was believed to provide adequate typing of class I HLA molecules for medical and scientific applications. Systematic molecular analysis of the polymorphism has however revealed limitations in the aerological methods. Many alleles remain undiscovered, particularly in Africans and other non-European populations. Even in the most extensively studied populations new subtypes continue to emerge. The degree of accuracy to which alleles can be assigned serologically is variable, as is the extent to which patterns of aerological cross-reactivity correlate with the underlying structures of the proteins. The problems are obvious in the case of HLA-C, but apply similarly to HLA-A and B.

A fundamental requirement for analysis of the effects of HLA-A, B, C type on T-cell vaccination, whether aimed at infectious, autoimmune and malignant diseases, or on transplantation between unrelated donors and recipients, is typing that distinguishes all alleles with comparable levels of accuracy. This requirement is most likely to be met by DNA-based methods and development and application is one goal of the next international histocompatibility workshop. Another goal is to focus on human populations in which the HLA profile is poorly defined. It is hoped that the latter effort can be integrated with that of the Human Genome Diversity Project which proposes to provide samples from hundreds of anthropologically characterized human populations.

HLA-A, B, C Genes and Natural Selection

In contrast to HLA-A, B and C the class I MHC pseudogenes, HLA-H and HLA-J, show very little polymorphism (Zemmour *et al.*, 1990; Messer *et al.*, 1992). Thus function and polymorphism are correlated, suggesting that polymorphism in the HLA-A, B and C genes is the result of natural selection. This idea is supported by the nature and distribution of nucleotide substitutions within the genes; coding substitutions affecting residues of the antigen recognition site that contact bound peptides are disproportionately represented (Parham *et al.*, 1988; Hughes and Nei, 1988; Hedrick *et al.*, 1991). Furthermore, it is clear from many studies of peptide binding and antigen presentation that amino acid changes at such 'peptide-binding positions' affect the specificity of the binding site. All these data are consistent with the view that polymorphism is naturally selected to give molecules with different specificity for the presentation of antigens.

Disease-specific Selection

Survival depends on T-cell responses against an array of micro organisms, viruses and foreign proteins. That individuals with any pair of HLA-A, B and C alleles survive suggests that all the alleles present in the human population are capable of presenting a wide range of antigens, a conjecture supported by the diversity of endogenous peptides that are bound by the product of a single allele (Jardetzky *et al.*, 1991). Over and above these basic requirements an individual allele may offer a particular advantage under specialized circumstance, for example, when a particular disease is a major threat to survival. It would be envisaged that alleles that are better at presenting peptides from the disease-causing pathogen would be favoured under these circumstances. The high frequency of HLA-B53 in West African regions of endemic malaria provides an example of such disease-specific selection (Hill *et al.*, 1991, 1992). In many other parts of the world disproportionately high frequencies of particular alleles and haplotypes are found (Imanishi *et al.*, 1992) and are candidates for being associated with resistance to particular disease, but the connections between host and pathogen have yet to be made.

Overdominant Selection (Heterozygote Advantage)

Peptides bound by class I molecules are usually nine amino acids in length. The ends of the peptides are bound tightly through 'anchor' residues, which are often conserved amongst the peptides bound by a particular allele (reviewed in Barber and Parham, 1993). In contrast the centre of the peptide is less important for interaction with HLA and appears to define the epitope seen by the T-cell receptor. The peptides bound by different alleles are distinct. For example, at position 2 of peptides bound by B27, B7 and B60, arginine, proline and glutamic acid residues respectively are found (Hill *et al.*, 1992, unpublished

data). Thus, from a given antigenic protein each of these molecules will present a different set of epitopes to T.cells.

In view of the mechanism of peptide presentation the potential benefit of heterozygosity is obvious. Heterozygotes can present a greater array of peptides than homozygotes and by inference should be capable of making a stronger immune response against any pathogen. The maximum twofold effect will most closely be approached when there is no overlap between the repertoire of peptides bound by the two alleles. Thus one might expect a tendency for populations to evolve alleles that are highly distinctive and to lose intermediate forms. This effect could explain the presence of distinctive lineages of HLA-A alleles and the segregation of closely related subtypes amongst populations (Kato *et al.*, 1989; López de Castro, 1989).

Frequency Dependent Selection

So far we have considered two components of the selection upon Class I HLA molecules. Disease-specific selection, which assesses the ability of class I molecules to present a limited number of defined peptides, and overdominant selection which assesses their capacity to present a universe of peptides.

Let us consider a hypothetical population in which overdominance is the only selection and all alleles, though complementary in their binding site specificities, are as effective in their function of antigen presentation. Under these circumstances selection will act to even the frequency of alleles, thereby maximizing the proportion of homozygotes. This same tendency will confer selective advantage to new alieles providing they too are complementary to existing alleles and as functionally effective. Most new alleles are formed by conversions that reassort clusters of substitutions between functional alleles. They are likely to be functionally distinct and the presence of many such products in human populations suggests they are functionally effective. The prevalence of previously undiscovered HLA-B alleles in South American Indian populations (Belich *et al.*, 1992; Watkins *et al.*, 1992) indicates that the appearance of new alleles is sufficiently frequent to be a significant factor in considerations of the selection acting upon class I HLA genes.

New alleles are advantageous not because they are better than existing alleles but because they are different; this is a frequency-dependent selection. As the frequency of a new allele increases under this selection its selective advantage will decrease, eventually to zero when the new allele has reached a frequency comparable to that of the existing alleles. The selective advantage of a new allele will be dependent upon the number and distribution of existing alleles, and thus upon population size. For large urban populations, with their many alleles and few homozygotes, this advantage should be considerably less than for small tribal populations with limited numbers of alleles and greater homozygosity.

Many of the HLA-B alleles found in South American Indians are not present in North American Indians or Japanese, two genetically related populations. The new HLA-B alleles can be derived from HLA-B alleles found in North American populations by single conversion events and this is probably how they evolved. Despite the production of new alleles the numbers of HLA-B alleles in South American Indian populations do not appear to be greater than those in

North American Indian groups (Belich *et al.*, 1992; Watkins *et al*., 1992). Thus the introduction of new alleles has been accompanied by the loss of the existing alleles. This may be due to genetic drift, which has increasing impact with decreasing population size; modern populations of South American Indians are small. As a new allele increases in frequency in a population the frequencies of the existing alleles inevitably decrease and the chance of them being lost commensurately increases. The replacement of old alleles by new ones could also result from disease-specific selections and the environment of South America certainly harbours different pathogens from that of North America.

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Summary of discussion

Chairperson: A. Teale
Rapporteurs: S. Kemp and J. -R. Scheerlinck

The possibility of IFNγ production by bovine *T. parva* specific $CD8^+$ cells and its potential role in protection was discussed. Although secretion of IFNγ by this population has not been examined, a direct role of this cytokine during infection was considered unlikely. Although in vivo and indirect effects cannot be excluded, it has been established that recombinant IFNγ does not affect the growth of *T. parva*-infected cells *in vitro.* Several mechanisms were suggested to explain the non-reciprocal cross-protective capacities of the Muguga and Marikebuni stocks of the parasite. The possibility of differential sensitivity of the stocks to CTL activity was suggested but epitope dominance during the course of the immunizing infection was considered a more plausible explanation. Since individual identical twins immunized with Muguga and Marakebuni stocks can develop strain-specific and cross reactive responses respectively, it was considered unlikely that host MHC differences can account for this phenomenon.

The potential for peptide immunization as a means of generating of *T. parva-specific* CTL was discussed. The low affinity of CTL induced in this manner was highlighted as a major drawback. An additional concern was the observation that the optimal size for the immunization is longer at 16aa than the 9aa preferred for optimal binding of peptides te the MHC binding cleft. This may reflect an extended half life of the longer peptides in *vivo.* Other issues raised in this context were the possibilities that the absence of $CD4^+$ T-cell epitopes in short peptides used to induce CTL might lead to the induction of sub-optimal responses, and that immunization with high doses of peptide might induce energy rather then stimulation.

The possibility that polymorphism in TAP.and proteosome molecules in different cell types might contribute to the selection of class I MHC-bound peptides was discussed in relation to the selection of target cells for the identification of bovine CTL epitopes using gene transfection of heterologous cells expressing bovine class I MHC antigens. Glycosylation of antigens was also relevant in this regard. Glycosyl moieties might be expected to obstruct the association of a given peptide with the antigen binding groove, so that removal of glycosylation sites could result in the generation of new T-cell epitopes. More importantly, because glycoproteins are co-translationally translocated to the endoplasmic reticulum, they would not be expected to access the proteosome/TAP.processing pathway.

The use of peptides stripped from class I MHC molecules on the surface of APC to identify CTL epitopes was discussed at length. The type of APC used and the dilution at which the crudely separated peptides can be used (around 103 to 104 should be considered as a minimum) were highlighted as being of

crucial importance to the success of the technique. The use of the mouse RMA-S mutant cell line transfected with a bovine class I MHC gene was considered a good option for the identification of bovine CTL epitopes, since these cells present peptides 10 times better then the parent line RMA. Prediction of epitopes on the basis of the presence of an MHC binding motif alone was discouraged, even in the case of mice, since many motif-containing peptides will bind to class I MHC molecules without constituting T-cell epitopes.

In discussion of MHC polymorphism and its possible influence on the efficacy of a vaccine targeted at the induction of CTL, it was emphasized that care should be taken to maintain heterozygosity of the MHC when selecting for particular traits in cattle populations. This increases the chance that the population would survive infection with previously unencountered pathogens. The concept of selecting cattle with MHC genes that interact well with vaccine antigens is therefore flawed. The aim should be to produce a vaccine capable of providing protection in cattle of many different MHC types. It was recommended that, once identified, a candidate vaccine antigen for the induction of *T. parva*-specific CTL should be tested in a genetically diverse population of cattle.

IMMUNOPATHOGENESIS: PARASITE-INDUCED DETRIMENTAL IMMUNE RESPONSES

An anti-disease vaccine for malaria?

J.H.L. Playfair

Department of Immunology
UCL Medical School, London, UK

Several lines of argument suggest that it might be worth considering a malaria vaccine that operates against the disease rather than the parasite itself. There is a long literature devoted to the concept of 'toxins' in clinical malaria and the development of anti-toxic immunity in endemic areas and standard vaccines against well-established toxins (e.g. tetanus, diphtheria) are among the most effective that exist. More recently, attention has focussed on the possible toxic role of cytokines, particularly tumour necrosis factor (TNF) in severe *Plasmodium falciparum* malaria, and on the identification of the parasite components that induce them. There is also the fact that solid immunity against the parasite is still, after decades of work, proving quite difficult to induce.

ANTI-TOXIC IMMUNITY

Early in the century, malaria was widely considered a toxic disease, and it was recognized that not all patients with the same parasitaemias were equally ill. Thus the simple presence of parasites was not sufficient to cause symptoms, which must therefore be produced by indirect means. Furthermore it was proposed that children could become immune to these toxic effects, and thus suffer less severe symptoms, at an earlier age than they acquired immunity to the parasite (Sinton *et al.*, 1931; Taliaferro, 1949; Hill *et al.*, 1943). More recent studies in the Gambia confirmed that clinical 'tolerance' (defined as 'the ability to live asymptomatically with fairly dense parasitaemia') developed by the age of three to five despite the persistence of high parasitaemias for up to five more years (McGregor *et al.*, 1956). No evidence has been produced that this tolerance was immunological in origin and it is still possible that it represents a reduced responsiveness to unchanged levels of some stimulating factor, or the presence of some natural blocking factor. However, recent work in experimental models, reviewed below, does suggest that acquired immunity to symptom-inducing antigens, possibly acting through TNF, might be part of the explanation.

In considering malaria from this viewpoint, it is necessary to distinguish several patterns of disease. Fever, the hallmark of malaria, is prominent in both *P.falciparum and P.vivax* infections, and there is quite good evidence linking it to over-production of TNF (Kwiatkowski *et al.*, 1993; see below). Anaemia, another almost universal complication, is particularly a feature of the first year of life, whereas cerebral malaria, the major cause of mortality, tends to occur from about a year later and is not seen in *P. vivax* infections. Nor is

hypoglycaemia, which is frequent in *P. falciparum* infection. Thus it is hardly likely that a single pathogenetic mechanism is responsible for all the clinical effects of the disease.

CYTOKINES

Nevertheless, as Clark was the first to point out (Clark, 1987; Clark *et al.*, 1989), there are theoretical arguments for an involvement of TNF in these and many other complications of *P. falciparum* malaria, which bear a striking resemblance to the toxic effects of high-dose TNF administration, and it is this cytokine that has been most thoroughly studied in this regard. There is a general consensus that raised plasma levels of TNF are associated with severity and mortality in cerebral malaria (Kem *et al.*, 1989; Grau *et al.*, 1990; Kwiatkowski *et al.*, 1990). In a mouse model that somewhat resembles cerebral malaria, anti-TNF antibody protected against cerebral disease but not against anaemia, but so did antibodies against several other cytokines (Grau *et al.*, 1988). In a small trial of anti-TNF antibody in human malaria, a reduction in fever was the only significant effect noted (Kwiatkowski *et al.*, 1993). Fever and raised TNF levels are also found in *P.vivax* infection (Karunaweera *et al.*, 1992), so if TNF is really responsible for cerebral malaria, some co-factor(s) must be involved too; a different pattern of sequestration could clearly be one of these, or there may be synergy with other cytokines or mediators. Interestingly, additive or synergistic effects are also seen in the anti-parasitic activity of TNF: killing of gametocytes *in vitro* appears to require TNF plus a 'complementary factor' (Nautunne *et al*., 1990). Although there is clearly much to be learned about both the pathogenetic and the protective activities of even this cytokine alone, it is important trying to understand why it is over-produced in malaria.

TNF-INDUCING MOLECULES

In experiments with the mouse parasite *P. yoelii*, we found that parasitized red cells incubated overnight with peritoneal macrophages induced amounts of TNF comparable to those induced by bacterial LPS. Both lethal and non-lethal strains of the parasite were active. We then found that incubating the parasitized blood over-night yielded supernatants that themselves induced TNF (Bate *et al.*, 1988). The human parasite *P. falciparum* behaved similarly, and either mouse macrophages or human blood monocytes could be used interchangeably (Taverne *et al.*, 1988). When injected into mice, these supernatants induced TNF sufficient to be detectable in the serum, and were lethal in mice that had been made hypersensitive to TNF by pre-treatment with D-galactosamine (Bate *et al.*, 1989).

Evidently, then, there were molecules in or on parasitized red cells that induced TNF production in macrophages. One obvious possibility was that the effect was the result of contaminating bacterial lipopolysaccharide (LPS) but, for reasons to be summarized later, we believe that this was not the case. However the active material did resemble LPS in certain respects; it retained activity after boiling, digestion with proteases and deamination by nitrous acid, but was destroyed by lipase treatment, by some types of phospholipase C, by

dephosphorylation with HF and by deacylation with NAOH. In a two-phase lipid extraction, it appeared in the chloroform/methanol rather than the water/methanol phase. When injected into normal mice at sub-toxic doses, it induced antibody which blocked its ability to induce TNF both *in vitro* and *in vivo*, and was predominantly IgM; we found no evidence for a memory response or a switch to IgG on boosting (Bate *et al.*, 1990). Using this assay, active material from all the species we have tested so far, including the mouse parasites *P. yoelii* and *P. berghei* and the human parasites *P. falciparum and P. vivax*, show complete cross-blocking, which is in striking contrast to the species, strain and even variant specificity of the protein antigens commonly studied (Bate *et al.*, 1992a). We concluded that parasitized red cells contain, and can release *(in vitro* at least), phospholipid molecules that induce TNF and behave as T-independent antigens. We refer to these molecules as parasite 'exoantigens' or 'toxic antigens' (Bate *et al.*, 1992b). Similar induction of TNF from human macrophages by *P. falciparum* supernatants has also been reported (Picot *et al.*, 1990).

Further characterization of the active molecule came from studies in which we used standard phospholipids of the type found in red cell membranes: phosphatidylcholine (PC), phosphatidyl serine (PS), phosphatidyl inositol (PI) and phosphatidyl ethenolamine (PE), as well as phosphatidic acid (PA) and cardiolipin (CL). None of these molecules induced TNF from macrophages, but one of them, PI, completely blocked the ability of exoantigens to induce TNE Surprisingly, the phosphated monosaccharide inositol monophosphate (IMP) also blocked, and so did preparations of exoantigen treated with either lipase or HF; we refer to these as 'detoxified'exoantigens (Bate *et al.*, 1992c). At this point we concluded that a phosphate group and an inositol ring were somehow involved in the TNF-triggering part of the exoantigens. This was further supported by the finding that antisera raised by simply immunizing mice with PI or IMP, but not the other phospholipids, blocked TNF release. However PC, PS and PA incorporated into liposomes with or without cholesterol did induce blocking antibody, but this could be absorbed out by PC liposomes, while antibody raised to PI or IMP.could not (Bate *et al.*, 1992d). From this we concluded that blocking antibodies were of two types—one specific for phosphate groups and one additionally recognizing inositol.

DISTINCTION FROM LPS

Our exoantigens appear to differ from LPS in a number of important ways. First, their TNF-triggering activity is not blocked by the inclusion of polymyxin B in the cultures, while the blocking antisera, whether against the exoantigens themselves or against PI or IMP, do not block triggering by LPS. Second, they stimulate macrophages from the LPS-hyporesponsive C3H/HeJ mouse strain. Third, triggering by LPS is not abolished, as that of the exoantigens is, by phospholipase C digestion; conversely deamination abolished the activity of LPS but not of the exoantigens. Fourth, neither PI, IMP.or detoxified exoantigens block triggering by LPS, which may even suggest that different macrophage receptors are involved. We therefore feel safe in concluding that LPS alone cannot account for our results. However we cannot eliminate the possibility that trace amounts of LPS, insufficient to stimulate on their own, are

needed as 'adjuvant' to the effects of the exoantigens, particularly where induction of blocking antibody is concerned (Schuster *et al*., 1979; Banerji and Alving, 1990); indeed there would be nothing unphysiological if this was so, since malaria patients probably have increased amounts of circulating LPS (Felton *et al.*, 1980).

HYPOGLYCAEMIA

During the experiments on toxicity of exoantigens *in vivo*, we had noticed that injected mice often looked ruffled and ill. Investigating this, we found a dramatic drop in blood sugar lasting from two until at least eight hours after injection. By comparison with the normal mid-morning blood sugar of 7.6 mmol/L, the four-hour value averaged 3.4 mmol/L, and values below 2 mmol/L were quite common. Since TNF (and other cytokines) can cause hypoglycaemia, we attempted to inhibit this drop with repeated injections of a monoclonal anti-TNF antibody. Though this antibody protected D-galactosamine pre-treated mice from death, it had no effect on hypoglycaemia. On the other hand, a second and third injection of exoantigen at two-week intervals induced progressively less hypoglycaemia in normal mice, but this protection was not seen in mice with Severe Combined Immunodeficiency (SCID mice). We concluded that antibody against the exoantigens could protect against hypoglycaemia. In line with the TNF experiments described earlier, we then showed that IMP.mixed with the exoantigens blocked their hypoglycaemic effect, as also did antibody raised against IMP.or against a PI-BSA conjugate (Taylor *et al.*, 1992).

We do not know whether the same molecules) induced both TNF and hypoglycaemia. Moreover we have occasionally detected low levels of 'TNF and hypoglycaemia induction with supernatants of normal red cells, so that it remains possible that the active phospholipids are normal red cell components that are increased in concentration or otherwise modified by the parasite (Hsaio *et al.*, 1991; Vial *et al.*, 1990).

ANAEMIA

Anaemia is recognized as one of the most severe complications of malaria, particularly in very young children, and it is generally agreed that it cannot be accounted for solely by red cell parasitization (Abdalla *et al.*, 1980). In our non-lethal *P.yoelii* mouse model, the haematocrit falls to 20% of normal in 10 days, despite the fact that the parasite is restricted to reticulocytes. The same is true in SCID mice, ruling out a major role for antibody. From about day 8 of infection, 51 Cr-labelled normal red cells are eliminated from the circulation unusually rapidly. Neither this effect, nor the anaemia itself, are prevented by prior immunization with exoantigens, nor by anti-TNF antibody, though TNF has been shown to depress erythropoiesis (Johnson *et al.*, 1989).

RELEVANCE TO THE INFECTION

Having shown that interesting and potentially toxic molecules are released by parasitized red cells *in vitro*, we wished to establish whether they were released during the infection. Here the evidence is incomplete. On the one hand, we do not detect TNF in the blood of infected mice, at least using the bioassay, and infected mice do not develop hypoglycaemia until about one day before they die; this might argue against a significant release of exoantigens *in vivo.* On the other hand, serum from infected mice does contain antibodies that block TNF induction by exoantigens, and also antibodies that bind PI and other phospholipids, which at least suggests that the immune system has been exposed to exoantigens. This paradox might be resolved if antibody produced early during infection was able to block both TNF induction and hypoglycaemia, and a detailed study in SCID mice would probably be necessary to test this. But another explanation that we have found attractive is that normal serum contains non-antibody blocking factors that would be capable of binding and neutralizing exoantigens until their concentration became overwhelming, analogous to, though not necessarily the same as, the lipoproteins that have been reported to inactivate LPS (Emancipator *et al.*, 1992). Supporting this idea, we have found that normal mouse serum does in fact block TNF induction by exoantigens, but only at dilutions up to about 1120; by comparison, the titres of antibody in immunized mice frequently exceed 1/10,000.

RELEVANCE TO PROTECTIVE IMMUNITY AND VACCINATION

The analysis of exoantigens that induce TNF and/or hypoglycaemia would be rather academic if they could not be exploited as protective vaccines. At present this is far from being the case, but there are some encouraging pointers. In a large group of genetically homogeneous Fl hybrid mice immunized with exoantigens and challenged 13 days later with the uniformly lethal *P.yoelii*, about half recovered after about three weeks with very high parasitaemias (in some mice over 80%) without looking particularly ill. This is, of course, what 'anti-disease' immunity would be expected to look like. Similar protection has been obtained in smaller groups of mice immunized with PI and IMP. Neither repeated boosting nor the use of adjuvants improves this level of protection, and though preliminary experiments with exoantigens coupled to protein carriers such as bovine serum albumin and keyhole limpet haemocyanin have yielded substantial increases of blocking antibody, most of which is IgG, the level of actual protection remains the same (Bate *et al.*, 1993). Perhaps IgM is the more effective isotype in this case.

This may simply reflect the fact that our *P. yoelii* model is not really suitable for this type of protection study. For example, as noted above, both vaccinated and non-vaccinated mice become extremely anaemic during infection, and it may be that mice are dying of anaemia though 'protected' against other aspects of pathology. This is typical of the difficulties encountered when trying to study 'anti-disease' immunity in such a multifactorial disease. However in a model of cerebral malaria using C57BL mice and *P. berghei,* immunization with exoantigens has been shown to give 60% protection against early ('cerebral') mortality (Curfs *et al.*, 1992) and a somewhat similar partial protection with exoantigens has also been reported in a *P. falciparum* model in squirrel monkeys (Ristic and Kreier, 1984), and in cattle with babesiosis (James, 1989).

RELEVANCE TO THE HUMAN DISEASE

Our hypothesis, then, is that the 'anti-toxic' immunity observed in human populations is due to the development of immunity against exoantigens, probably mediated by antibody which blocks their ability to induce TNF, hypoglycaemia etc. This will be hard to prove, but one could make certain predictions. 'Tolerant' children with high parasitaemia but mild or no symptoms ought to have blocking antibody in their blood; we are beginning to test this. In immune adults who leave the endemic areas, clinical immunity (IgM?) might be expected to wane more rapidly than immunity to the parasite itself (IgG?), and this does often seem to be the case. If we are right about the conserved nature of the toxic exoantigens, anti-toxic immunity should show a wider degree of cross-protection than anti-parasite immunity, but this is still controversial (Jeffery, 1966). Finally, antibody against the relevant exoantigens ought to correlate with clinical protection. One such exoantigen, a TNF-inducing molecule found in *P.falciparum* culture supernatants and originally named Antigen 7 by its discoverers, fits the latter criterion since levels of antibody to this antigen peak in Gambian children at about the age of five, which is when clinical immunity develops; antibody to most other exoantigens peaks considerably later (Jakobsen *et al.*, 1991). Moreover, T-cell responses to Antigen 7, assayed by IFNG release, correlate with clinical severity, which might be important in view of the synergy between IFNG and TNF-inducing molecules (Riley *et al.*, 1991). Unfortunately the best correlation with clinical immunity in individual patients was with antibody to another exoantigen (Antigen 2). This kind of analysis is further complicated by the fact that several of these antibodies also seem to affect parasitaemia. Careful and substantial studies of TNF and other cytokine responses to a range of exoantigens, in the presence of sera from both immune and non-immune patients, will be necessary before the existence of true 'anti-toxic' immunity in man can be established. Nonetheless whether or not it exists in the natural state, the ability to induce it by immunization could be of great potential value.

RELEVANCE TO HUMAN VACCINES

As compared to the candidate vaccines already under study, the anti-disease approach has both advantages and disadvantages. In its favour is the fact that it aims to deal with what really matters—disease. Also, the apparent lack of antigenic variation noted in the mouse experiments might suggest that a whole series of species and variant-specific antigens would not be needed. Moreover by acting against cytokine-inducing antigens rather than against the cytokines themselves, as, for example, antibody to TNF or soluble TNF receptors do, it might exert an effect on other cytokines induced at the same time, such as IL1, and thus avoid the need for a complex 'cocktail' of inhibitors. Against it is the lack of T-dependent antibody, with memory, IgG etc., in the mouse experiments which, if translated directly into the field, would mean a vaccine injection every few weeks, or at best a 'tourist' vaccine. Another theoretical danger is that the phospholipid antigens responsible for disease might turn out to be genuine autoantigens, or at least cross-react with host molecules; the vaccine would then induce autoimmunity. We are currently attempting to confer T-dependence on the relevant antigen, in the hope that this might possibly overcome both these problems.

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Production of tumour necrosis factor α during bovine trypanosomiasis: possible correlation with severity of anaemia associated with the disease

M. Sileghem and L. Gaidulis

International Laboratory for Research on Animal Diseases
P.O. Box 30709, Nairobi, Kenya

INTRODUCTION

Activation of cells from the monocyte/macrophage lineage during African trypanosomiasis has been reported for experimental model infections in inbred mouse strains (Grosskinsky *et al.,* 1983) and tsetse-transmitted infection in cattle (Flynn and Sileghem, 1991; Sileghem *et al.,* 1993). Although the exact impact of this macrophage (MO) activation on the immune system is not entirely understood, it has been suggested that it might be responsible for a variety of immune disorders including immunosuppression and polyclonal activation (Askonas, 1985; Sileghem *et al.,* 1989). Furthermore, activation of MO might also have a direct impact on the disease. Cachexia, a state of exhaustion attributed to MO-derived cytokines (Beutler and Cerami, 1986; Franks *et al.,* 1991) is a prominent feature of trypanosomiasis (Rouzer and Cerami, 1980; Morrison *et al.,* 1981) and uptake of erythrocytes by activated MO has long since been accepted as a major cause of anaemia associated with the disease (Connal, 191 1; Fiennes, 1954; Murray and Dexter, 1988). MO activation during African trypanosomiasis would thus appear to have deleterious rather than a beneficial effect for the host.

In a variety of disease models, erythrophagocytosis, dyserythropoiesis, cachexia and polyclonal activation have been attributed to tumour necrosis factor a (TNFα) (Clark and Chaudri, 1988; Miller *et al.,* 1989; Beutler and Cerami, 1986; Macchia *et al.,* 1993), a cytokine that is predominantly secreted by cells of the monocyte/macrophage lineage. TNFα has also been implicated in the immunosuppression that is characteristic of African trypanosomiasis (Lucas *et al.,* 1993). Production of TNFα might thus represent a central mechanism through which activated MO can mediate a variety of the effects reported during trypanosomiasis. It was, therefore, important to determine whether TNFα production is induced during bovine trypanosomiasis and whether or not production is correlated with disease.

PRODUCTION OF TNFα DURING BOVINE TRYPANOSOMIASIS

To measure TNFα during infection in cattle, we have developed a capture immunoassay (EIA) using a mouse-anti bovine TNFα monoclonal antibody for capture (BC9, Sileghem *et al.,* 1992) and a rabbit polyclonal anti-bovine TNFα antiserum for detection. TNFα production was studied in cattle infected with *T. vivax* IL2337 by tsetse fly inoculation (Moloo *et al.,* 1987). This isolate induces acute anaemia and marked erythrophagocytosis in cattle (Anosa *et al.,* 1992). Plasma TNFOC was measured weekly by the EIA using recombinant bovine TNFα (rTNFα) diluted in pooled pre-infection plasma as a standard. The limit of detection was defined as three times the standard deviation of the optical density from the background (pooled pre-infection plasma), which was 200 pg/ml. Small amounts of the cytokine were recorded only on days 14 to 21 pi (Figure 1). In cattle, plasma TNFα titres have been shown to be increased within hours of intravenous administration of bacterial lipopolysaccharide (Peel *et al.,* 1990) or of intratracheal infusion of *Pasteurella haemolytica (Pace et al.,* 1993). However, plasma TNFcc titres remained negative throughout Gram negative septicaemia (Peel *et al.,* 1990). Circulating cytokines represent the excess of cytokine synthesis and therefore may not accurately reflect the production of a given cytokine during disease (Cavaillon *et al.,* 1992). We have therefore resorted to *ex vivo* measurements of TNFα activity. Peripheral blood mononuclear cells (PBMC) were sampled from three animals prior to, and following, tsetse fly transmitted infection of *T.vivax* IL2337. Monocytes were purified by adhesion to plasma-coated gelatine (Goddeeris *et al.,* 1986) and cultured. TNF in 5 hr and 24 hr culture supernatants was measured using a modification of the EIA described in which standards were diluted in culture medium instead of plasma. The use of culture medium instead of plasma as a negative control resulted in a decrease of detection limit to 100 pg/ml. TNF was present in 5 hr supernatants derived from all three animals studied (Figure 2A) although titres varied considerably from individual to individual. Production was maximal at day 17 pi and fell rapidly thereafter; the cytokine was not detected in the 24 hr supernatants (data not shown).

Because cytokine production *ex vivo* does not necessarily reflect the amount produced *in vivo,* care should be taken in interpreting the results. However, *ex vivo* titrations can indicate the capacity of cells to produce cytokines and have been useful in relating production of interleukin-1 to various human disease states (reviewed by Dinarello, 1991). In our experiments, monocytes prepared at various time points were all cultured in the same batch of medium. Therefore, the absence of TNFα production in monocytes prepared before infection and those isolated after week 4 pi would argue against these results being an *in vitro* artefact and would suggest that the production of TNF *ex vivo* is due to *in vivo* macrophage activation.

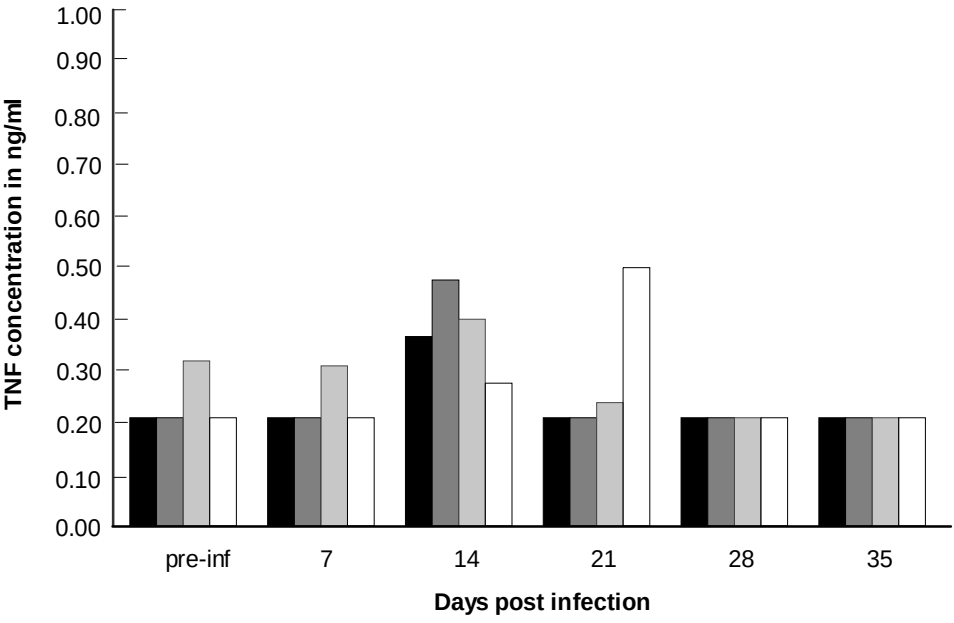

Figure 1. TNFα titres in plasma from Boran cattle infected with *T.vivax* IL2337 by tsetse fly transmission. At weekly intervals, blood from four animals was sampled into ethylene-diaminetetra-acetate tubes for preparation of plasma and the samples were stored at -70 ?C until use. TNFα was titrated by a capture EIA using a mouse monoclonal anti-bovine TNFα antibody (BC9) and, for detection, a rabbit polyclonal anti-bovine TNF antiserum. The rabbit antibody was detected by incubation with biotin-conjugated donkey anti-rabbit immunoglobulin (Amersham International, Amersham, Bucks, UK), and streptavidin-conjugated horseradish peroxidase (Amersham). Following addition of 2,2?-azino-bis(3-ethylbenz-thiazoline -6-sulfonic acid) (ABTS), the optical densities were determined at 414 nm in a Titertek Multiskan MCC 340 (Flow Laboratories). Pooled pre-infection plasma was used as the negative control and bovine rTNFα, diluted in pooled pre-infection plasma, was used to generate a standard curve. The limit of detection, defined as the optical density from the negative control $^{+}$ 3 times the standard deviation, was 200 pg/ml. The four bars represent four individual animals.

ASSOCIATION OF TNFα PRODUCTION WITH SEVERITY OF DISEASE

When monocytes were purified from cattle infected with *T.congolense* ILNat 3.1 (Nantulya *et al.*, 1984) rather than *T.vivax* IL2337, no *ex vivo* TNFα production was detected (Figure 2B). These parasites were selected for this study because of the marked difference in anaemia associated with the infections they induce. The *T.vivax* stock IL2337 causes acute anaemia, early severe erythrophagocytosis and a progressive reduction in packed cell volume (PCV) that reaches a minimum around week 2 to 3 pi (Anosa *et al.*, 1992). At that

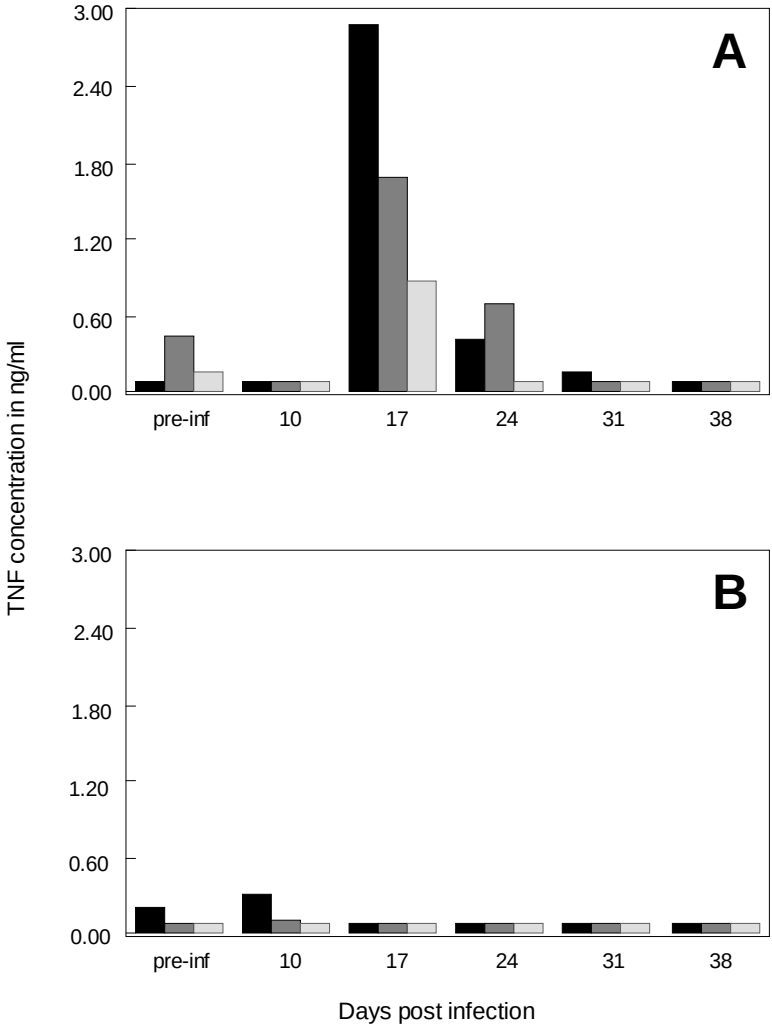

Figure 2.TNFα titres in culture supernatants of monocytes. At weekly intervals following infection by tsetse fly inoculation, blood was sampled from three Boran cattle infected with *T. vivax* IL2337 (A) and from three Boran cattle infected with *T. congolense* ILNat 3.1 (B). The different bars represent individual animals. The blood was collected into Alsever?s solution, PBMC were prepared by density centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) and monocytes purified from the PBMC by adhesion to plasma-coated gelatin surfaces. The monocytes were cultured in 100 µl aliquots in 96 well flat bottomed plates (Costar, Cambridge MA) at a concentration of 10_6/ml in RPMI 1640 (Flow Laboratories, Irvine, UK) supplemented with 5% heat-inactivated foetal calf serum (Flow Laboratories), 2mM L-Glutamine and 50 units/ml penicillin-streptomycin (culture medium). The cells were incubated for 5 h at 37 ÉC in a humidified atmosphere containing 5% CO_2. The supernatants were stored at -70 ºC until use. TNFα was titrated by a capture EIA as described in Figure 1 with the only modifications being the use of culture medium as a negative control and bovine rTNFα diluted in culture medium instead of plasma to prepare the standard curve. The limit of detection was 100 pg/ml

time, some animals seem to overcome the acute anaemia, and their PCV values increase. Many animals, however, require trypanocidal drug treatment to avoid death from anaemia. In contrast, erythrophagocytosis is less marked during infection with *T. congolense* ILNat 3. 1, and the PCV is generally stable until week 2 pi, when it begins to fall reaching a minimum around week 6 to 7 pi. At this point, some animals recover and proceed to the chronic stage of the disease. Most animals, however, require treatment (Paling *et al.*, 1991). Anaemia is thus observed during infection with both parasites, but is much more acute and severe during infection with *T .vivax* IL2337.

Although it is not clear whether the differences in TNFα production between the two infections are quantitative or qualitative, our data demonstrate unambiguously that *ex vivo* TNFA secretion by monocytes is higher during infection with *T.vivax* IL2337 than during infection with *T.congolense* ILNat 3. 1. To establish whether the *ex vivo* observations were a true reflection of the *in vivo* situation, RNA was extracted from monocytes purified on magnetic beads through negative selection to avoid activation. TNFα mRNA was then detected using bovine TNFα-specific oligonueleotide primers in a 35 cycles reverse transcriptase polymerase chain reaction. TNFα message was detected in monocytes from both *T.congolense*- and *T.*vivax-infected animals, but was much higher in the *T.vivax-in*fected animals early in infection (Table 1). Thus, the differences between the two groups are apparent at the mRNA level, but are quantitative rather than qualitative.

In conclusion, TNFα production is induced during bovine trypanosomiasis and is higher in *T.vivax* IL2337 infection than in *T.congolense* ILNat 3.1 infection. This difference is observed at the level of *ex vivo* protein secretion and at the level of *in vivo* mRNA expression. Since TNFα has been proposed as the cause of erythrophagocytosis in murine malaria (Clark and Chaudri, 1988), it is interesting that a correlation is found between TNFα production and erythrophagocytosis in bovine trypanosomiasis. This does not establish a causative role for TNFα in erythrophagocytosis, but the association of TNFα production with the severity of anaemia and erythrophagocytosis makes this cytokine a clear candidate for future research into the pathogenesis of bovine trypanosomiasis.

Table 1. TNFα mRNA expression in monocytes isolated from *Trypanosoma vivax* IL2337 and *T.congolense* ILNat 3.1 infected cattle.

	Pre-infection	Day 3 pi	Day 10 pi
T.congolense ILNat3.1			
BJ296	-	-	-
BJ300	-	-	-
BJ301	-	-	++
BJ349	-	-	++
T.vivax IL2337			
BJ249	+	++	++
BJ250	-	++	++
BJ344	-	++	++
BJ352	-	ND	++

Blood was sampled from eight Boran cattle prior to and following tsetse fly-transmitted infection with either *T.*vivax IL2337 or *T.Bongo* ILNat3.1 and collected into Alsever's solution. PBMC were purified by density centrifugation on Ficoll-Paque. T.cells were removed using magnetic beads (Dynal, Oslo, Norway) coated with anti-CD2, anti-CD4 and anti-CD8 monoclonal antibodies, γδ T.cells using beads coated with an anti-WCI antibody and B cells using an anti-lgm monoclonal antibody. The negative population was found to contain over 80% monocytes (IL-A24-positive). RNA was extracted by the one-step method of Chomezynsky and Sacchi (1987) and 1 µg was reverse transcribed into single stranded(ss) cDNA using AMV reverse transcriptase and oligo(dt)15 as the primer (Promega, Madison, WI), according to the manufacturer's directions. One tenth (5 µl) of the total cDNA mix was used as a template in a PCR reaction containing 1.5 mM $M9Cl_2$, 15 pM of each primer, 2.5 U Taq Polymerase and 200 µM of each dNTP. The primers used were CATCAACAGGCCTCTGGTCAG sense and GAGTCCGGGCAGGTCTACTTTGGGATCATTGCCCTGTGA antisense. The 498 bp product was amplified through 35 cycles wiTh1 minute denaturing at 95°C, 1 minute annealing at 55°C and 2 minutes extension at 72°C. All samples were tested for the presence of beta actin prior to the TNFα analysis to ensure that cDNA synthesis and amplification was possible from all the samples. A negative control containing all components except cDNA and a positive control of LPS stimulated alveolar macrophages was included in every analysis. The PCR products were evaluated by electrophoresis through an ethidium bromide containing 1.2% agarose gel. This figure represents a composite of a visual analysis of the agarose gels.

INDUCTION OF TNFα PRODUCTION *IN VITRO* BY RYPANOSOMAL ANTIGENS

When monocytes and tissue macrophages from uninfected cattle were stimulated *in vitro* with bacterial lipopolysaccharide (LPS), tissue macrophages were found to secrete high titres of TNFα whereas the secretion of TNFα by the monocytes fluctuated around the detection limit of our assay. However, when the monocytes were primed with bovine interferon g prior to stimulation with LPS, secretion of TNFα was up to 2000 times higher than background levels. Monocytes therefore proved more suitable indicators of the macrophage-activating potential of parasite fractions than tissue macrophages since they allowed the distinction of priming and trigger functions.

When a *T .congolense* lysate was added to freshly isolated monocytes no TNFα production was induced. The lysates were then tested in the presence of erogenous triggers or priming agents to further assess their MO activating potential. After being cultured for 24 to 48 h with a trypanosome lysate, monocytes failed to respond to an external trigger. However, when monocytes were primed by incubation with interferon γ for 24 h, a strong TNFα secretion was observed after the addition of *T. congolense* lysate. These results indicate that *T. congolense* lysates are unable to prime monocytes but are able to trigger primed cells. The parasite factors responsible for this stimulation are currently being purified.

CONCLUDING REMARKS

African trypanosomes live free in the bloodstream where they are a direct target for the host antibodies. Since antibodies are predominantly directed to the surface coat, the capacity of the parasite to change its coat provides it with a powerful mechanism to escape immune elimination. In addition, the vertebrate host does not acquire an immune status after parasite clearance through drug treatment. Since African trypanosomes infect a variety of domestic and wild animals that form a reservoir from which new infections can be established, it is clear that drug treatment cannot provide a long-term solution to trypanosomiasis. For these reasons, the need for prophylactic measures has often been put forward.

Due to the extreme variability of the exposed epitopes of VSG molecules, the development of a conventional anti-parasite vaccine based on the induction of anti-surface coat antibodies is not a feasible option. Therefore, alternative strategies for disease control are being explored such as the development of anti-disease vaccines. Our observations that *T. congolense* molecules directly activate macrophages to produce TNFA and that TNFα production during disease might be correlated with severity of anaemia provide new options for the design of alternative control measures. Whether preventing TNFα production would be sufficient to protect infected animals from the disease and whether the TNFα-inducing molecule will ever be suitable as a vaccine candidate still remain open questions. Nonetheless the development of an anti-

disease vaccine based on TNF-inducing factors clearly deserves further exploration.

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Protective and inappropriate antibody responses in trypanosome-infected cattle

D.J.L. Williams, K. Taylor, J. Newson, J. Naessens and E. Authié

International Laboratory for Research on Animal Diseases
P.O. Box 30709, Nairobi, Kenya

INTRODUCTION

The trypanosome surface coat is composed almost entirely of a homogeneous array of molecules known as variable surface glycoproteins (VSG). These molecules are highly immunogenic and elicit a strong antibody response in the infected host. Antibodies against the surface-exposed epitopes of a particular VSG mediate the destruction and clearance of trypanosomes expressing that variant (Morrison *et al,* 1982). As those parasites are cleared, they are replaced by others expressing antigenically different VSG molecules, which in turn provoke specific antibody responses. Trypanosome infections are therefore characterized by waves of parasitaemia followed by waves of VSG-specific antibody.

It is known that trypanotolerant N'Dama cattle infected with *Trypanosoma congolense* have significantly lower levels of parasitaemia than the more susceptible Boran cattle (Paling *et al,* 1991 a). It has been suggested that this reflects a more effective VSG-specific antibody response in the N'Damas (Murray *et al.,* 1982). We have tested this hypothesis by examining VSG-specific antibody responses in N'Dama and Boran cattle during experimental infection with *T.congolense.*

RESULTS AND DISCUSSION

Eleven cattle of each species were infected using tsetse flies *(Glossina morsitans centralis)* that had fed on a goat infected with *T.congolense* ILNat 3. 1, according to the method of Dwinger *et al.* (1987). The course of infection was monitored by measuring percentage packed red cell volume (PCV) and estimating parasitaemia by dark ground/phase contrast microscopy (Paris *et al.,* 1982) and serum samples were collected from each animal at weekly intervals. Cattle whose PCV fell to 15% were treated with diminazene aceturate.

All cattle became parasitaemic between 10 and 12 days after infection (dpi). The average parasitaemia in the Boran group during the eight-week monitoring period was higher than that of the N'Dama group, whereas the mean PCV of the Borans was lower than that of the N'Damas. Although the PCV fell to 15% in

eight of the eleven Borans, that of all N'Damas remained above this value throughout the experiment.

Serum antibodies to VSG epitopes exposed on the surface of the parasite were measured in a complement lysis assay using live *T. congolense* ILNat 3.1 parasites. Lytic antibodies were detectable in serum from all cattle by day 21 of infection, and in one Boran animal had appeared by day 14. Antibodies persisted until conclusion of the analysis at day 42. There was no significant difference in lytic activity of sera from the two groups.

The isotype distribution of antibodies specific for surface-exposed epitopes of VSG was determined in sera from four N'Damas and four Borans, by FACS analysis of live trypanosomes incubated with heat inactivated bovine test sera and stained with monoclonal antibodies (MAb) specific for bovine immunoglobulin isotypes. Specific antibodies of IgM, IgG, and IgG_2 isotypes were present in all sera, and the kinetics of their appearance and their titres were similar in both groups. Highest titres of IgM antibodies were observed on day 21 in all eight cattle and these had disappeared by day 35 of infection. Antibodies of IgG_1 and IgG_2 isotypes were first detected 21 dpi and were still present in sera taken on day 49.

Formalin fixation disrupts the trypanosome surface coat and reveals epitopes on VSG molecules that are not accessible on the surface of living parasites. Antibodies that bound to formalin fixed trypanosomes were measured using an ELISA technique (Voller *et al.*, 1975; Williams *et al.*, 1990). A trypanosome-specific IgM response was observed using this assay in both N'Damas and Borans that peaked at day 21 and declined thereafter. No significant difference was apparent between the two groups in the intensity of this response. Although trypanosome-specific IgG, was also detected in both groups from day 14, titres were significantly higher in the N'Dama group. No specific antibodies of IgG_2 isotype were detected in either group of cattle.

To examine the development of non-specific antibody responses in cattle following infection with *T.* congolense, antibodies specific for *Escherichia coli* b-galactosidase were measured in sera from each group using an ELISA assay. No b-galactosidase-specific antibodies were detected in sera collected from the N'Dama cattle at any time. In contrast, responses to this antigen were detected in the Borans between days 14 and 35 after infection, with highest titres being observed on day 21; these antibodies were all of the IgM class. b-galactosidase-specific IgM was prepared from the sera by affinity chromatography with MAb ILA30 (Naessens *et al.*, 1988) followed by a second chromatography step using a b-galactosidase affinity column. By testing the bound and unbound fractions in the trypanosome ELISA described above, it was established that the b-galactosidase specific antibodies in these sera bind trypanosome antigens.

CONCLUSIONS

The results of these experiments demonstrate that antibody responses made by N'Dama and Boran cattle against surface-exposed epitopes of VSG during primary infection with *T. congolense* are quantitatively and qualitatively similar. Trypanotolerance in N'Dama cattle is therefore not related to a more effective antibody response against the trypanosome surface coat.

Further analysis revealed that although Boran cattle make strong IgM responses to non-conformational epitopes of VSG, IgG_1 responses to these specificities were poor in comparison to those observed in the N'Dama group. In addition, IgM responses in the Boran cattle appeared polyspecific in nature. These observations are indicative of a defect in isotype switching and affinity maturation in Boran cattle that is not apparent in the trypanotolerant N'Damas. Since both of these functions are T-cell dependent, our results are consistent with a poor helper T-cell response in *T. congolense-infected* Boran cattle.

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B-cell activation in trypanosome-infected Boran cattle

K.A. Taylor, D.J.L. Williams and V. Lutje

International Laboratory for Research on Animal Diseases
P.O. Box 30709, Nairobi, Kenya

INTRODUCTION

Analysis of serum antibody responses in cattle experimentally infected with *Trypanosoma congolense* suggested that antibody responses of trypanosusceptible Boran cattle are less efficient in isotype switching and affinity maturation than those of trypanotolerant N'Dama cattle (see preceding report by Williams *et al.;* Authie *et al.,* 1993). Briefly, high levels of polyspecific IgM are produced by Boran cattle while N'Dama cattle have higher titres of IgG specific for certain trypanosome antigens.

Further characterization of these differences required determination of the frequency and phenotype of antibody secreting cells (ASC), as well as the specificity of the antibody and the isotype secreted in response to infection. A method was therefore developed to maintain bovine B cells in culture and stimulate them to differentiate into ASC. B cells were examined for the expression of the transferrin receptor and the CD5 antigen using fluorescence activated cell sorter (FACS) analysis and the number of ASC was determined using an ELISpot assay.

We compared B-cell activation and the frequency of ASC between six *Trypanosoma congolense-infected* cattle and three uninfected controls. Peripheral blood lymphocytes (PBL) were analysed before and after *in vitro* culture for three and six days with combinations of lipopolysaccharide (LPS), pokeweed mitogen (PWM) and recombinant bovine interleukin-2 (rboll 2). Splenic lymphocytes from *T. congolense-infected* cattle were compared with PBL from the same animals.

CHARACTERIZATION OF B CELLS

The total number of B cells and their expression of the transferrin receptor and the CD5 antigen was determined by FACS analysis. The percentage of B cells in freshly isolated PBL was significantly higher in infected (60.1%) than in uninfected animals (25.6%) (Figure 1). After three days *in vitro* stimulation the percentage of B cells present in cultures from uninfected animals was unchanged under all culture conditions. However, the percentage of B cells was significantly higher in cultures from infected animals stimulated with LPS (79.0%) and LPS plus rboIL 2 (72.8%), than those maintained with medium

alone (47.5%). The expression of the transferrin receptor on B cells from PBL of uninfected and infected animals was 4.0 and 3.3% respectively (Figure 2). The percentage of transferrin receptor positive B cells was significantly greater than the medium control in cultures from cells of infected animals stimulated for three days under all conditions. The highest expression (44.1%) was seen in cells stimulated with LPS plus rboll 2.

The level of transferrin receptor expression was compared between B cells derived from PBL and spleen of infected animals. The mean percentage of total B cells derived from the PBL (54.1%) was not significantly different from that found in the spleen (48.8%). However, the level of transferrin receptor expression on B cells from the spleens (22.7%) was significantly higher than from PBL (5.6%).

DIFFERENTIATION OF NON-SECRETING B CELLS TO ANTIBODY-SECRETING CELLS

The number of ASC was determined by ELISpot assay. No ASC were ever detected in freshly isolated PBL. PBL from uninfected animals had a very low frequency of ASC after *in vitro* stimulation for three days (Figure 3). B cells from infected animals differentiated to ASC when stimulated for three days with LPS (0.32%), LPS plus rboll 2 (0.17%), rboIL 2 (0.06%) and PWM (0.05%). The percentage of ASC decreased significantly when rboIL 2 was included with PWM (0.0 1 %). After six days *in vitro* stimulation all responses were enhanced, particularly among the uninfected animals, where frequencies approached those seen with infected animals (data not shown).

Unlike B cells from PBL, fresh splenic B cells secreted antibody *ex vivo* (Figure 4). In addition, three days stimulation was sufficient to obtain the maximum number of ASC as opposed to six days for the PBL.

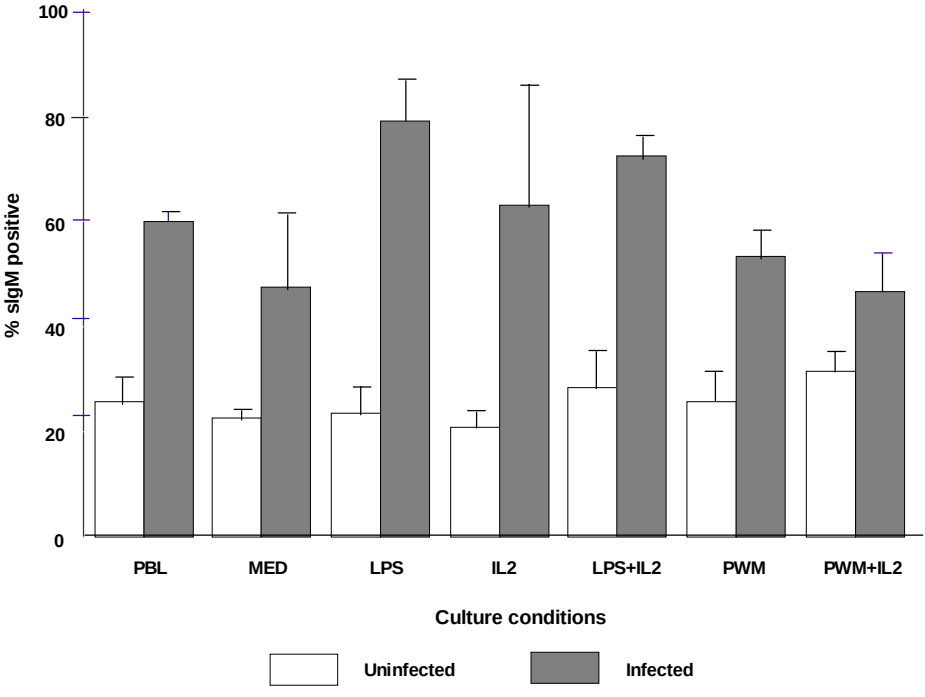

Figure 1. Mean percent of B cells measured by MAb IL-A 30 (anti-IgM) in the peripheral blood of uninfected (n=3) and *Trypanosoma congolense*-infected (n=3) Boran cattle before (PBLs) and after three-day *in vitro* culture under various conditions.

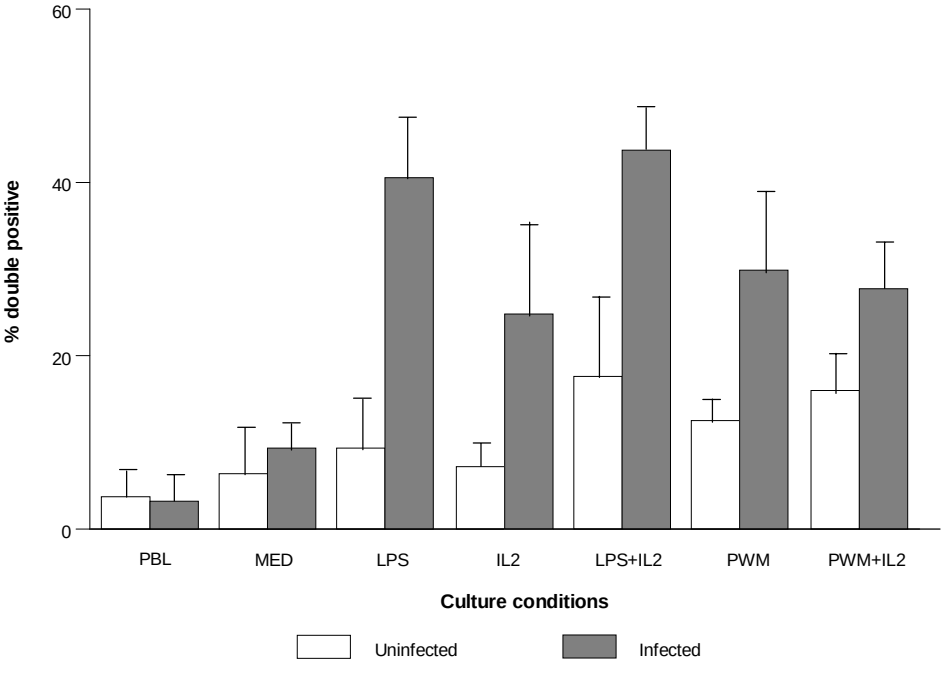

Figure 2. Mean percent of transferrin receptor-positive B cells measured by MAb IL-A77 and IL-A30 in the peripheral blood of uninfected (n=3) and *T. congolense*-infected (n=3) Boran cattle before and after three-day *in vitro* culture under various conditions.

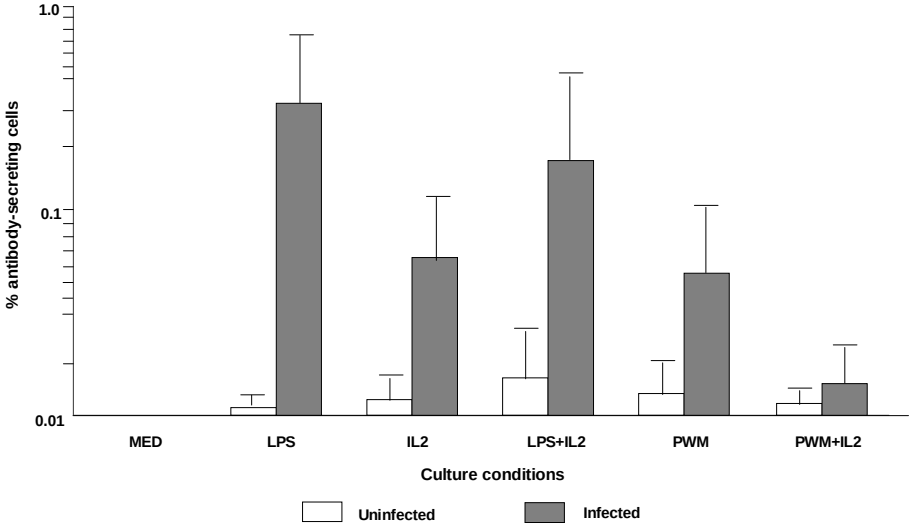

Figure 3. Mean percent antibody secreting cells from peripheral blook of uninfected (n=3) and *T. congolense*-infected (n=6) Boran cattle after three-day *in vitro* culture under various conditions.

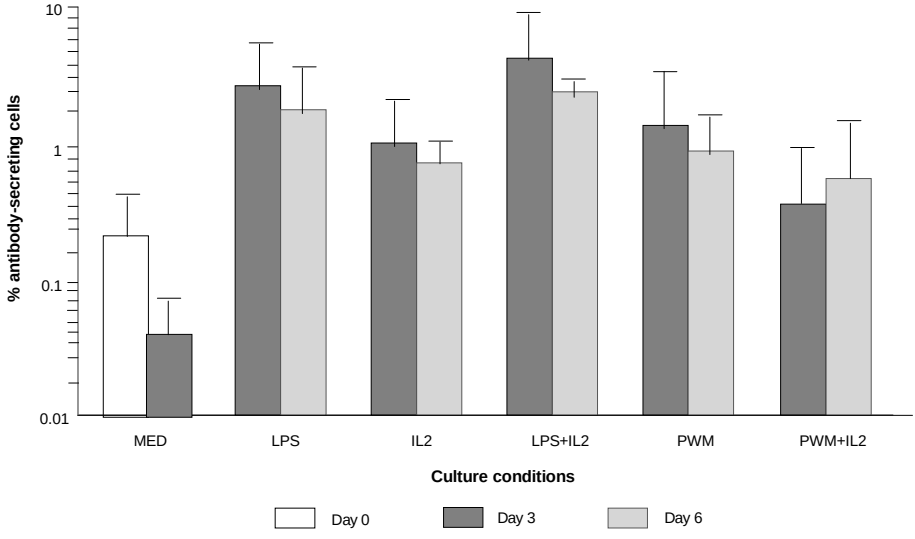

Figure 4. Mean percent antibody secreting cells from spleens of *T. congolense*-infected (n=4) Boran cattle before and after three- and six-day culture under various conditions.

CONCLUSIONS

The use of LPS, PWM and IL2 to stimulate B cells to secrete antibody *in vitro* and detection of ASC by an ELISpot assay has been thoroughly described in the mouse and human systems. However, information on the activation of bovine B cells is limited. There are reports of stimulating bovine B cells with PWM or human IL2 (Olobo and Black, 1989; Lutje and Black, 1992; Collins and Oldham, 1993). In these cases activation of B cells was measured either through the Ig content of the culture supernatants or plaque assays measuring responses to sheep red blood cells. Here we have described a comparative analysis of *in vitro* stimulation of bovine peripheral blood and splenic lymphocytes from healthy and trypanosome-infected cattle. The differentiation of B cells with respect to expression of an activation marker, the transferrin receptor, and their ability to secrete immunoglobulin, was quantitated using FACS analysis and an ELISpot assay.

An increase in the number of circulating B cells during trypanosome infection in cattle has previously been reported by Williams *et al* . (1991). Cattle infected with *T. congolense* have a higher frequency of circulating B cells that can be stimulated *in vitro* to express the transferrin receptor and secrete antibody than uninfected cattle. LPS and LPS plus IL2 were the most potent stimulators of both transferrin receptor expression and antibody secretion. Stimulation with either PWM or IL2 alone resulted in an increase in the number of ASC; however when both stimulators were present there were fewer ASC compared to activation with either stimulus alone. This phenomenon was also observed for human peripheral blood cells by Sauerwein *et al* . (1986).

The level of activation of B cells found in the spleen differed from those found in peripheral blood. A greater number of B cells from the spleen expressed the transferrin receptor and some are capable of secreting antibody *ex vivo* without further stimulation. When stimulated *in vitro,* splenic B cells require only three days for the maximum number of cells to differentiate to ASC as opposed to six days for B cells from the peripheral blood.

This work will now be extended to compare the isotype and specificity of antibody produced by trypanotolerant and trypanosusceptible breeds of cattle in response to trypanosome infection.

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Bovine T-cell responses to defined *Trypanosoma congolense* antigens during infection

V. Lutje, E. Authié. A. Boulangé and D.J.L. Williams

International Laboratory for Research on Animal Diseases
P.O. Box 30709, Nairobi, Kenya

INTRODUCTION

Infections of cattle with tsetse-transmitted *Trypanosoma congolense* are accompanied by antibody responses to the variable surface glycoprotein (VSG) and to two major invariant antigens, a member of the hsp70 family (B oulang6 and Authié,1994) and a 33 kDa cysteine protease (CP; Authié *et al .,* 1992). During primary infection, trypanotolerant N'Dama *(Bos taurus)* cattle exhibit higher levels of IgGl to CP than susceptible Borans *(Bos indicus;* Authié *et al,* 1993a). Boran cattle, on the other hand, have high levels of IgM to irrelevant antigens. During rechallenge infections, N'Dama cattle have higher levels of IGGI to hsp70 while Boran cattle exhibit high titres of IgM to this antigen. Although Boran cattle do generate high titres of IgM te hsp70, N'Dama cattle develop higher levels of specific IgG antibody (Authi6 *et al.,* 1993b). These observations are consistent with a possible dysfunction in isotype switch from IgM to IgG in Boran cattle. Because of the central role of T helper cells in induction of Ig isotype switch, the difference in antibody responses between N'Dama and Boran cattle might reflect defective T helper cell function in the susceptible breeds during infection.

We undertook a study to analyse T-cell function in cattle during infection with *T. congolense* . T-cell proliferative responses to a recombinant form of the hsp70 (R63), cysteine protease and VSG were measured in a group of Boran cattle following primary challenge with *T. congolense* . The role of $CD4^+$ and $CD8^+$ cells in the response was also investigated.

RESULTS

No proliferative responses to the CP, R63 or VSG were detected in cultures of PBL from cattle infected with *T. congolense* ILNat 3.1 up to 45 days post-infection (data not shown). However, in three animals tested on day 4, 11 and 19 post-challenge, respectively, all cells obtained from lymph nodes draining the site of infection proliferated in the presence of all three antigens (Figure 1).

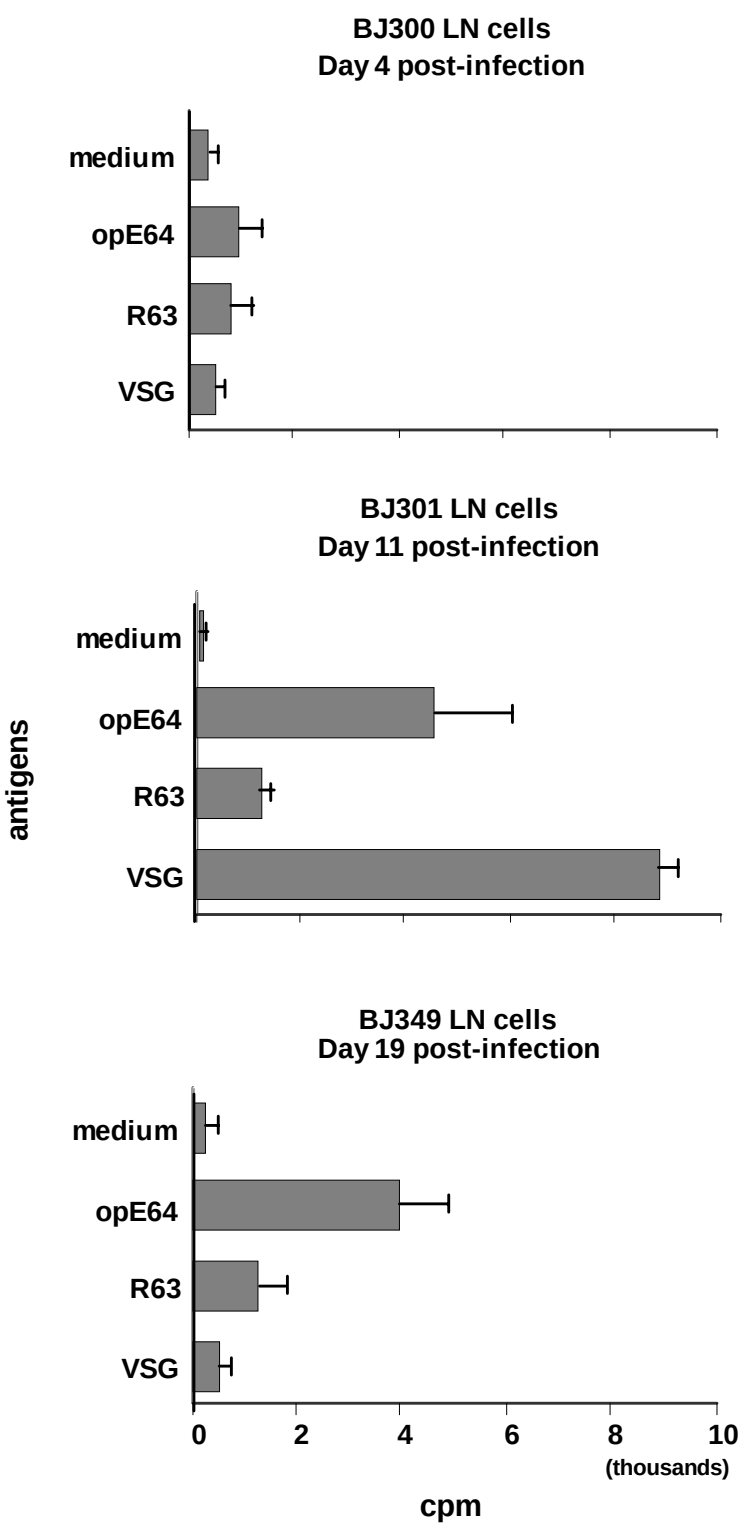

Figure 1. Proliferative responses of LN cells from three Boran cattle at different stages of primary infection with *Trypanosoma congolense* to the cysteine protease (cpE64), recombinant 63 kDa protein (R63) and VSG.

Proliferative responses to the CP, R63 and VSG were highest in LN cells from animal BJ30 1, which was tested 11 days after challenge. When LN cells from another animal, BJ349, were tested 19 days after challenge, significant proliferative responses to the CP and to R63 were detectable. This experiment was repeated in a different group of animals and similar results were obtained, with highest proliferative responses observed in LN cells from day 11 post-infection (data not shown).

The role of $CD4^+$ and $CD8^+$ cells in the response was analysed by their selective depletion through FACS sorting after staining with specific MAbs. No proliferative responses to the CP or R63 were detected after depletion of either $CD4^+$ or $CD8^+$ T cells from whole LN cell populations (Figure 2). The response to the VSG was abrogated by the depletion of $CD4^+$ cells but unaffected by the removal of $CD8^+$ cells.

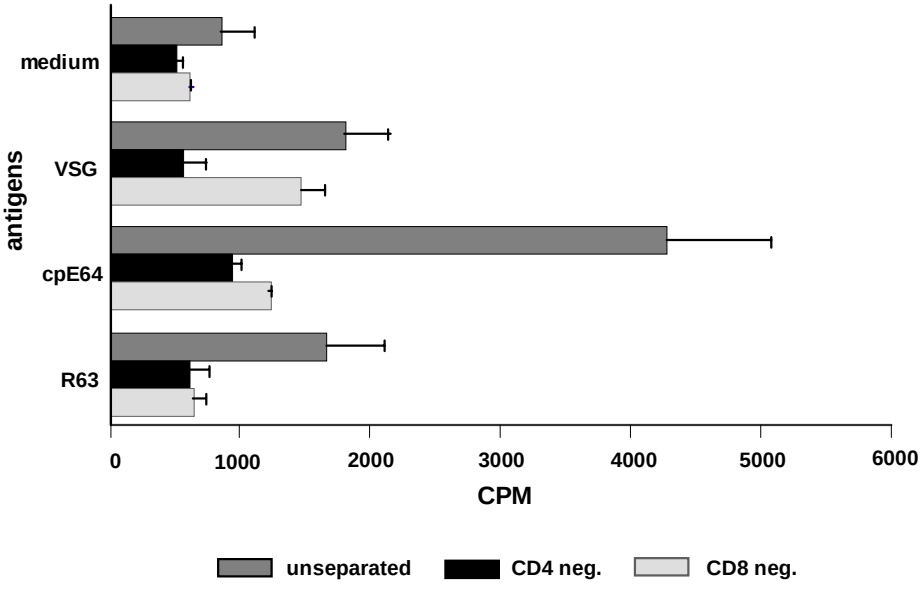

Figure 2. Role of $CD4^+$ and $CD8^+$ LN cells in the proliferative response to *Trypanosoma congolense* antigens.

DISCUSSION AND CONCLUSIONS

In this study we demonstrate, for the first time, that T cells respond to three immunodominant antigens of *T. congolense* , i.e. cysteine protease, hsp70 and VSG, in lymph nodes from Boran cattle undergoing primary infection. A previous study (Flynn *et al* ., 1992) reported that no proliferation to trypanosome antigens could be detected in bovine lymph node cells during primary challenge. However, whole trypanosome lysate was used as an antigen, and only one animal per week post-infection was tested. Interpretation of that study may therefore have been complicated by variation in the intensity of individual animal responses. Furthermore, in our hands, T-cell responses to

trypanosome lysate were generally poorer than those observed with the defined antigens under discussion (data not shown).

By depletion of T-cell subsets, we showed that bovine cell responses to VSG involve mainly $CD4^+$ T cells. Whereas both $CD4^+$ and $CD8^+$ cells are required for the response to cysteine protease and hsp70. T-cell responses to VSG have not been detected so far in cattle following infection and have only recently been described in mice infected with *Trypanosoma brucei rhodesiense (Schleifer et al .,* 1993). The response in mice is only found in peritoneal T cells, which secrete TH1-like cytokines (gamma-interferon and IL2) but do not proliferate to VSG (Schleifer *et al,* 1993). T cells responding to a cysteine protease of *Trypanosoma cruzi* have recently been detected in chagasic patients (Arnholdt *et al,* 1993) and have been characterized as $CD4^+$ and T helper 1 -type (TH1). In our study, proliferative responses to cystein protease and invariant hsplo antigens were abrogated by the depletion of both $CD4^+$ and $CD8^+$ T cells. It is not clear as yet whether both $CD4^+$ and $CD8^+$ T cells are responding to these invariant antigens or whether they are mutually regulated by cytokine production. The difference in T-cell types responding to VSG and to two invariant antigens might be attributed to differences in antigen presentation. Further experiments are needed to clarify these issues.

The study reported here provides the first evidence of T-cell responses to defined antigens of *T. congolense* in cattle following tsetse-transmitted infection. We are currently undertaking more detailed experiments involving production of T-cell lines and cytokine analysis, in order to analyse the quality of T-cell help provided to B cells during infection, and to determine whether differences exist between T-cell responses of trypanotolerant and trypanosusceptible breeds that might account for differences in antibody isotype switch between the two breeds.

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$CD5^+$ B lymphocytes in cattle infected with African trypanosomes

J. Naessens

International Laboratory for Research on Animal Diseases
P.O. Box 30709, Nairobi, Kenya

Although specific immunoglobulin (Ig) and B-cell responses are suppressed during infections with African trypanosomes, a general activation of the humoral wing of the immune system is observed. The changes that occur in the humoral immune system during these infections have been reviewed (Sileghem *et al*, 1993) and can be summarized as follows:

- an increase in cellularity of the spleen and in the proliferative status of spleen cells;
- an increase in the numbers of circulating B cells;
- an increase in the levels of circulating Ig, particularly that of the IgM class;
- the appearance of increased titres of antibodies specific for antigens of non-trypanosome origin (heterophylic Abs, non-specific Abs);
- the presence of auto-antibodies and immune complexes.

The massive increase in the numbers of circulating B cells, Ig and the occurrence of antibodies to apparently unrelated antigens could be explained by polyclonal activation of B cells such as that provoked in murine B cells by bacterial lipopolysaccharide. Many studies have been undertaken to find which trypanosome product might be responsible for the polyclonal B-cell stimulation and the generation of non-specific antibodies (reviewed in Sileghem *et al* ., 1993). Although purified trypanosome fractions can be shown to induce antibodies to non-trypanosome antigens, no general consensus has been reached over which parasite antigen gives rise to the polyclonal B-cell activation seen during infection.

However, a subset of B cells known as the B-1 subpopulation, the majority of which express the Ly-1 or CD5 leukocyte differentiation antigen, has been described (reviewed in Herzenberg and Cantor, 1993; Haughton *et al.*, 1993) that is functionally associated with some of the features seen in trypanosome infection. B-1 cells are largely responsible for normal secretion of IgM and the secretion of autoantibodies and the subset is expanded during certain autoimmune diseases and chronic infections. We have therefore analysed the expression of CD5 by bovine B lymphocytes during the course of *Trypanosoma congolense* infections (Naessens and Williams, 1992).

In normal uninfected cattle $CD5^+$ B cells represent about 20% of mononuclear leukocytes in peripheral blood, 5-10% of those in the spleen, and 1-2% of lymph node cells. The population has not been observed in Peyer's patches, and no $CD5^+$ B cells have been detected in foetal blood and spleen. $CD5^+$ B cells of cattle have the same surface phenotype as their CD5- counterparts, apart from a higher mean concentration of membrane IgM, and are therefore similar to

human and murine B-1 cells. As in mouse and man, bovine $CD5^+$ B cells can be shown to express the integrin CD11b (Mac-1). $CD5^+$ B cells are slightly larger than classical B cells, and show higher forward and side scatter profiles on the flow cytometer.

In monitoring $CD5^+$ B-cell numbers in *T. congolense -infected* cattle, a sharp increase in the proportion of total circulating B cells was observed at around week 3 of infection, approximately one week after parasites were first detected in peripheral blood. The numbers of $CD5^+$ B cells rose steadily until they constituted 60-80% of the peripheral blood B-cell population. Analysis of the relative proportions of $CD5^+$ and CD5- B cells in these animals revealed that expansion in B-cell numbers was almost entirely due to the increase in $CD5^+$ component. The tissue-distribution of $CD5^+$ B cells remained the same as before infection with the largest numbers occurring in blood, somewhat fewer in the spleen, very few (less than 5%) in lymph node, and none being detectable in Peyer's patches. A significant difference in the behaviour of $CD5^+$ B cells during infection was not observed between susceptible Boran and resistant N'Dama cattle breeds. An increase in numbers of $CD5^+$ B cells has also been observed in cattle infected with *T. vivax.*

The raised numbers of $CD5^+$ B cells were kinetically associated with the observed increase in total serum IgM. It is therefore tempting to speculate that it is the expansion of the $CD5^+$ B population during infection that leads to the secretion of large amounts of IgM. By analogy with human and murine $CD5^+$ B cells, they may also be responsible for secretion of polyspecific Ig and autoantibodies and may explain the occurrence of high titres of antibodies with non-trypanosome specificities. It is therefore important to determine why these cells are preferentially amplified during trypanosome infections, and what signals, from parasite or host, contribute to their expansion.

It is important to note that it has not yet been shown definitively that bovine $CD5^+$ B cells are homologous to B-1 cells, and a number of observations may dispute the hypothesis. Prominent among these is that it has not been possible to demonstrate the expression of IgD by bovine B cells, using immunoprecipitations with monoclonal antibodies specific for IgM and Ig light chain on lymphocytes derived from a number of tissues. Because their lack of IgD distinguishes B-1 cells from classical B cells in mouse and man, this feature provides a difficulty in making a similar distinction in cattle. A further anomaly is that CD5 has not been found on B cells in the bovine foetus, whereas CDS^+B B cells constitute the largest B-cell population of human and murine foetal tissue. It remains a possibility that ruminants possess only B cells of the B - 1 class; this is known to be the case for the rabbit, in which all B cells express CD5. In this situation, it is possible that CD5 behaves as an activation marker. In the light of these observations, it is clearly important to establish whether $CD5^+$ B cells in cattle do behave as B-1 cells and give rise to the secretion of IgM and high titres of antibodies with non-trypanosome specificity during infection with African trypanosomes.

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Summary of discussion

Chairpersons: D. McKeever and M. Sileghem
Rapporteurs: P. Gardiner and L. Gaidulis

This session consisted of eight presentations on the perturbations of the immune system caused by immune response to parasite antigens. In the introduction, Dr. D. Williams (ILRAD) outlined two different areas of research concern to the trypanosomiasis program. One goal was to identify trypanosomal antigens responsible for the immunopathogenesis of the disease by determining how the infection of cattle with trypanosomes affects their immune system. A second goal was to identify protective immune responses that might be targeted at the parasite itself or at the mechanisms through which it causes disease. The first presentation by Dr. John Playfair (University College, London) focused on the anti-disease approach to a vaccine for malaria. In this type of a vaccine, parasite molecules, identified as phospholipid exoantigens, responsible for over-production of TNFα in murine malaria, were shown to protect against the clinical symptoms of the disease. He pointed out that while TNFα was responsible for fever and other complications of malaria, studies with injections of TNFα anti-serum in mice infected with *P. chabaudi* greatly reduced their parasitaemia. TNFα could in this model play both a protective and pathogenic role. A similar approach to an anti-disease vaccine for trypanosomiasis was presented by Dr. Maarten Sileghem (ILRAD).

He showed that *T. congolense* lysates contain macrophage-activating factors that are capable of suppressing T-cell proliferation in cattle. On the other hand, the membrane form of VSG, specifically the phosphatidyl inositol anchor, was a potent inducer of the cytokine TNFα, whose levels were shown to be positively correlated with the severity of anaemia. There were several presentations that centered on the role of the humoral response to trypanosomiasis (Dr. D. Williams, ILRAD), and especially on the importance of B-cell activation (Ms. K. Taylor, ILRAD) and the $CD5^+$ B-cell function both in trypanosomiasis and in Chaga's disease (Dr. J. Naessens, ILRAD; Dr. P. Nfinoprio, Institut Pasteur, Paris). Inefficient isotype switching and affinity maturation of antibodies, as well as a predominantly polyspecific IgM response to selected antigens were shown to be a characteristic of trypanosome infections in trypanosusceptible Boran cattle. Dr. Minoprio's presentation on the role of the two lymphocyte subsets, CD3 gamma-delta and $CD5^+$ B lymphocytes, showed clearly that they were involved in the autoimmune phenomena in the chronic phase of infection with T *cruzi*. Similarly, an increase in $CD5^+$ B cells during a trypanosome infection was demonstrated to occur in cattle by Dr. J. Naessens (ILRAD). The presentation by Dr. E. Authié (ILRAD) which highlighted differences between the two breeds of cattle in their immune response to two immunodominant trypanosome antigens, hsp70 and the 33 kDa cysteine protease (CP), identified two parasite antigens that could contribute to the pathogenesis of this disease. Data on T-cell function of trypanotolerant and trypanosusceptible cattle using

defined *T. convene* antigens was presented by Dr. V. Lutje (ILRAD) who showed that depletion of $CD4^+$ T cells or MHC class II^+ cells abrogated the proliferative response of lymph node cells to VSG *in vitro*, while depletion of either $CD4^+$ or $CD8^+$ cells abrogated the response to CP.

There were brief discussions following each presentation and the salient points were then summarized in a general discussion after the afternoon session. Chimaeric twins with bone-marrow derived cells originating partly from N'Dama and Boran breeds are an important resource at ILRAD for the analysis of mechanisms involved in trypanotolerance. A lengthy discussion followed as to how these animals could best be utilised for this purpose. One suggestion was that they would be useful for examining immunoglobulin class switching, since the capacity to effect this during infection might segregate with class II MHC type of the thymus-educated bone marrow cells. Since the chimaeras are stable and the twins are generally similar, varying only with the proportion bone marrow-derived cells of each genotype that they carry, it was considered that it might also be useful to isolate N'Dama and Boran $CD5^+$ B cells educated in the same environment to evaluate their respective function. Although 20–25 % of peripheral blood mononuclear cells in newborn cattle are $CD5^+$ B cells, very little is known about the function of this population, other than that its proportion increases to approximately 80% in adult cattle undergoing chronic infections. Since the immunoglobulin produced by these cells is polyspecific and of low affinity, it was suggested that they could conceivably serve as the first line of defence in a trypanosome infection, or as one of the earliest antigen presenting cells.

An attempt was made to identify the currently known differences in immune response between the breeds. The group concurred that in Boran cattle parasitaemia was always more marked, PCV was consistently lower, leukopenia was more pronounced; and that there was no IgG response to the cysteine protease (CP). These cattle also mount an IgM response to β-galactosidase, which is probably a reflection of polyspecific activation, while the N'Dama do not. Discussion then centered on identifying which traits or differences are not influenced by parasite load. Only the immune responses to the cysteine protease antigen and isotype class switching seemed to be candidates. Classical genetic studies on F2 generation of cattle using microsatellite markers to study linkage should eventually pinpoint these traits. The question was raised whether trypanotolerance results from a difference in parasite load or from different distribution of the parasite in the microenvironment. If the trait is reflected in a real difference in parasite load then control of growth of the parasite should be a focus of molecular vaccine research. A comment was made regarding the ability of buffalo to resist trypanosome infections. Like the N'Dama they appear to be able to mount a strong CP-specific response and, in addition, a host factor has been identified that is capable of lysing trypanosomes.

The major questions that arose from these presentations can be summarized as follows:

- Can trypanotolerance be explained in terms of parasite load or does it reflect breed differences in immune responses to trypanosome infection?

- Are perturbations of immunelcytokine networks the result of toxic molecules of the parasite or are they due to direct damage to host molecules and cell membranes?
- How does the quality of antibody responses in infection compare with that seen in response to purified antigens; can we alter specific responses to render them more appropriate to protection?
- By studying cytokine expression, antibody responses and toxic molecules of the parasite during infection, can we devise strategies to balance the immune system between suppression and exacerbation of pathology, or distinguish protective immune mechanisms from those that are detrimental to the host mechanism?

PROTECTIVE IMMUNE RESPONSES: T-CELL SUBSETS

T-cell subsets and cytokine profiles in the design of anti-protozoal vaccines

F.E.G. Cox and E.Y. Liew*[†]

* Division of Life Sciences
King's College London
London WS 7AH, UK

[†]Department of Immunology
University of Glasgow
Glasgow G 11 6NT, UK

INTRODUCTION

The conventional approach to vaccine design is the identification of dominant antigenic epitopes associated with protective immune responses and the construction of synthetic or recombinant versions of these epitopes in a form suitable for artificial immunization. This approach has been very successful in the design of vaccines against viral infections but has been less successful in protozoan infections, mainly because of the complexity of the responses against eukaryotic cells and the ability of the parasites themselves to manipulate the immune response for their own survival. Parasitic infections present their hosts with enormous immunological problems largely because of their antigenic complexity and complicated life cycles in which the various stages frequently differ antigenically from one another Parasites have also evolved numerous ways of evading the consequences of immune attack. Parasitic infections therefore tend to be chronic and accompanied by varied and unsuccessful immunological responses on the part of the host that frequently lead to immunopathological damage and immunosuppression. Until recently, our understanding of the immunity and immunopathology of parasitic infections has been hindered by the lack of a comprehensive and stable conceptual framework within which to work. This situation has been deterred by a number of discoveries that have led to the realization that both immunity and pathology are controlled by cytokines.

The first important discovery was that mouse $CD4^+$ lymphocytes could be divided into two functional subsets with distinct cytokine profiles-Thl largely dominated by the production of interferon-gamma, eliciting cell-mediated immunity, and Th2 largely dominated by the production of IL-4, IL-5 and IL-10, eliciting antibody-mediated immunity (Mosmann and Coffman, 1989). In parasitic infections these two immunological pathways are often mutually antagonistic with one being protective and the other counter-protective (Cox and Liew, 1992). Although the initial observations were made using inbred strains of mice it is now clear that analogous subsets occur in humans (Romagnani, 1991) and a search is on for similar T-cell populations in other species.

A general scheme of the basic features of cytokine activation that has stood the test of time is shown in Figure 1. Essentially, 711 cells produce two important cytokines-IL-2 that activates cytotoxic T cells and other lymphokine activated cells such as natural killer (NK) cells, and interferon-gamma that activates macrophages. Th2 cells produce IL-4, IL-5 and IL-10 that are responsible for the growth and differentiation of B cells and the production of antibody. There is a certain amount of cross cooperation, with the Thl product IL-2 being involved in the production of IgG2a, as well as antagonism with interferon gamma-inhibiting the production of antibodies and IL-4 and IL-10 inhibiting the activity of interferon gamma. However this simple pattern is continually being revised and added to as our understanding of the parts played by cytokines in infections increases and this has led to the creation of complex diagrams (Cox and Liew, 1992; Powrie and Coffman, 1993) and comprehensive charts (Burke *et al* ., 1993). Unfortunately, the demarcations between $CD4^+$ and $CD8^+$ activities and the roles of Th l and Th2 cells are breaking down as it becomes apparent that Th2 cytokines suppress the generation of $CD4^+$ cytotoxic cells and that $CD8^+$ cells regulate the production of Thl and Th2 cytokines. In parasitic infections, there is an additional complication in that cytokine profiles may vary during the course of an infection and even from host to host infected with the same parasite. Nevertheless, in parallel with investigations into the immunology of other infections, including AIDS, and cancers it is essential that both the general patterns and the complexity of the interactions between T-cell subsets and cytokines should be understood in parasitic infections.

CYTOKINES IN PARASITIC INFECTIONS

Although most of what we know about the role of cytokines in parasitic infections is derived from helminth infections (Finkleman *et al* ., 1991). This discussion will focus only on the general principles that apply to protozoal infections.

Protozoa that Live in Macrophages

Leishmania major infection of mice can be regarded as the basic model for these parasites and has provided the most information on the influence of cytokines in resolving and fulminating infections. It has also served as a baseline against which all other infections can be compared. In *L. major* infections in mice, interferon-gamma is clearly associated with recovery and/or resistance and IL-4 and IL-10 with exacerbated infections and/or susceptibility (Titus *et al* ., 1992; Liew and O'Donnell, 1993). In human infections, there is clear evidence that similar patterns occur. In *L. braziliensis* infections, interferongamma predominates in localized infections and IL-4 in destructive mucocutaneous

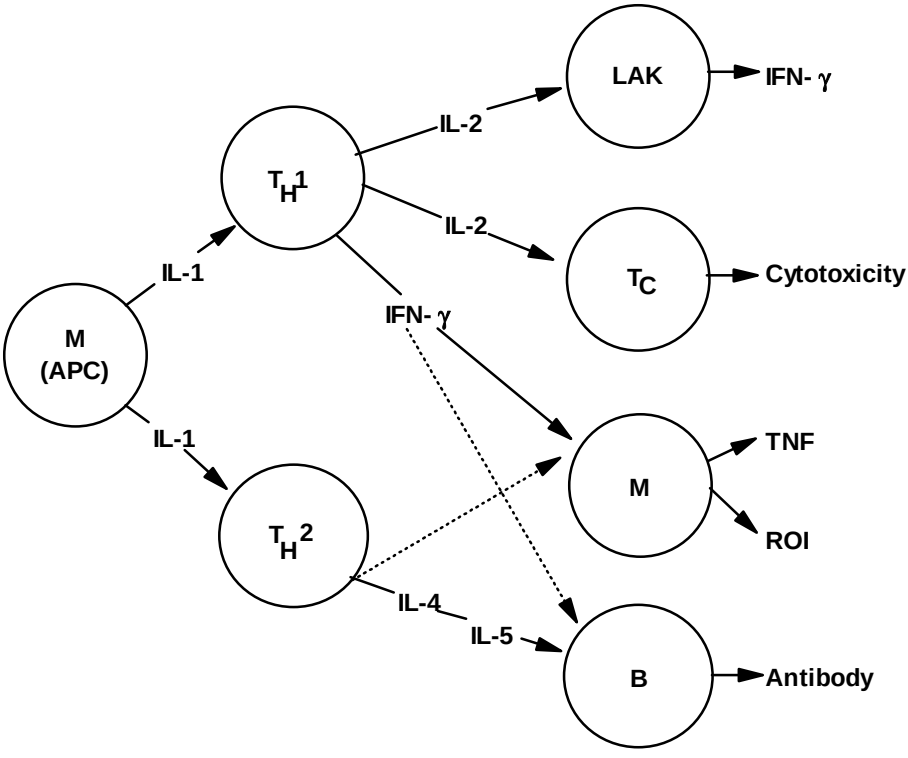

Figure 1. The overall pattern of cytokine involvement in defence against parasites. Activation is indicated by solid lines and inhibition by dashed lines.

forms of the disease (Pirmez *et al.*, 1993). In patients infected with *L. aethiopica, those* experiencing diffuse cutaneous leishmaniasis exhibit poor levels of IL-2 (Akuffo, 1992), suggesting an association with a defect in the Thl pathway. There is also evidence that interferon-gamma is indicative of disease limitation in *L. aethiopica* infections and that different parasite antigens elicit different patterns of cytokine response (Laskay *et al* ., 1991). In *L. chagasi* infections, patients with acute disease have lower levels of the protective cytokine interferon-gamma than those that were infected but showing little evidence of infection (Holaday *et al* ., 1993). In patients infected with *L. donovani, the* presence of IL- 10 correlates with the pathogenesis of the disease (Karp *et al* ., 1993).

Immunity of mice to *Toxoplasma gondii* also involves the Thl pathway and the production of interferon gamma (Beaman *et al* ., 1992). In humans, IL-2 and lymphokine activated natural killer cells are involved (Subauste *et al* ., 1992) suggesting that the Thl pathway is also important in human infections. However the development of a commercial live vaccine against toxoplasmosis in sheep based on the stimulation of antibody production (Buxton, 1993) only serves to indicate how much more information is required before a really effective and acceptable vaccine can be contemplated.

The situation in *Trypanosoma cruzi* infections is very confusing. IL-2 and IL-2 receptors seem to be the key molecules (Pakianathan and Kuhn, 1992) and $CD8^+$ T cells are important in controlling the infection at the cellular level (Sun

and Tarleton, 1993). There is also synergism between interferon gamma and tumour necrosis factor that results in the activation of trypanocidal mechanisms in the host macrophages (Munoz-Fernandez *et al.,* 1992). All this evidence supports a dependence on the Thl pathway, but IL-4, which might be thought to have an inhibitory counter-protective effect, also appears to play an important role in protection (Wirth *et al* 1989). An antigen specific IgGl response (suggesting a Th2 pathway) also seems to be important (Cerban *et al.*,1992). Taken together these observations, are difficult to explain in terms of the concepts outlined above.

Blood Parasites

The exhaustive studies of immunity to malaria has revealed no clear pattern of cytokine activity. Tumour necrosis factor and nitric oxide, both products of the Th1 pathway, appear to have a role in protection (Rockett *et al* .1992) but so do antibodies, which are products of the Th2 pathway.

The overall situation in animals infected with *Theileria* spp. is also unclear. It is widely accepted that $CD8^+$ cytolytic cells are important effector cells in recovery and immunity, and there are indications that helper and cytolytic $CD4^+$ cells may also be involved (Baldwin *et al* .1992). There is also evidence that macrophages, but not natural killer cells, are involved in immunity to *T annulata* (Fell and Preston, 1993).

Among the African trypanosomes, *T. b. brucei* stimulates $CD8^+$ cells that produce interferon gamma but also stimulate growth of the parasites (Olsson *et al* ., 1992). On the other hand, *T. b. brucei* and *T. b. gambiense* are susceptible to attack by activated macrophages (Vincendeau *et al* ., 1992).

This brief overview of the role of cytokines in protozoal infections, which has been drawn from some of the more recent literature, shows how little we understand the patterns of cytokine activity that occur during these infections and how difficult it is to make any all-embracing deductions. However, it should be possible, by systematically investigating both T-cell subsets and cytokine profiles, to obtain a very good idea about which components of the host reaction to protozoa are essential and protective and which are counter protective.

EFFECTOR MOLECULES

Examination of cytokine profiles in parasitic infections have provided information on the effector molecules involved. Antibodies, particularly IgM and IgGl, characteristic of Th2 cell activation, are involved, to a greater or lesser extent, in all infections except leishmaniasis. The effector molecules associated with the Thl pathway are mainly macrophage products, such as reactive oxygen intermediates, nitric oxide, and tumour necrosis factor; these molecules also contribute to the pathology associated with various parasitic infections (Cox and Liew, 1992). It is becoming clear that nitric oxide, with or without synergism with other cytokines or reactive oxygen intermediates, is a key molecule in parasite killing. The production of nitric oxide is controlled by an inducible enzyme, NO synthase, which is under the control of various cytokines;

interferon-gamma and tumour necrosis factor enhance its production and IL-4 and IL-10 inhibit it (Liew and O'Donnell, 1993)

RELEVANCE OF CYTOKINES

The study of cytokines is now central to our understanding of the immunology and immunopathology of parasitic infections; it is essential that we are able to distinguish between those cytokine responses that are protective and those that are deleterious. The results of such studies should lead to the possibility of the immune response towards protection and away from pathology and to the rational development of vaccines. This approach has already been suggested for non-parasitic infections and is well summarized in this quotation:

'Cytokines mediate communication within the immune system and between immune and other cell types. In contrast to antibody therapy, where a single agent can have a single defined effect, cytokine therapy relies on the subtle manipulation of the complex cytokine network, therefore presenting a major research challenge' (Gallagher *et al* ., 1993).

This challenge has already been taken up in general terms by Powrie and Coffman (1993) and it should now be possible for parasitologists to employ a rational approach to developing vaccines that elicit protective responses but not counter-protective or damaging ones.

CYTOKINES AND VACCINE DEVELOPMENT

We suggest a strategy for vaccine design which starts from the response itself and not the antigen. As pointed out above, immune responses are controlled and directed by various cytokines acting in concert or antagonistically and every infection induces a plethora of cytokines some of which are desirable and some of which are not. We now know that it is possible to characterize such cytokine profiles and to determine which are protective and which are harmful. Using this kind of information, it is possible to identify the T-cell subsets that are involved and so examine the actual nature of antigen presentation and processing that precedes the response. As particular epitopes drive the immune system towards either Th1 or Th2 responses, vaccines can be designed to facilitate an appropriate response.

The recognition of a particular epitope by the antigen-presenting cells which occurs as the result of interactions between the antigen-MHC complex and T-cell receptors is of considerable relevance to the design of successful vaccines. For example, transient carbonyl-amino condensations (Schiff base formation) between cell surface ligands occur and these can be finely tuned for the recognition of particular epitopes (Zeng *et al* ., 1992). Having identified desirable epitopes, amine-reactive carbonyl groups could therefore be manipulated to enhance the response to such epitopes at the expense of less desirable ones. It might also be possible to achieve the same ends by the selective use of adjuvants (Gordon, 1993).

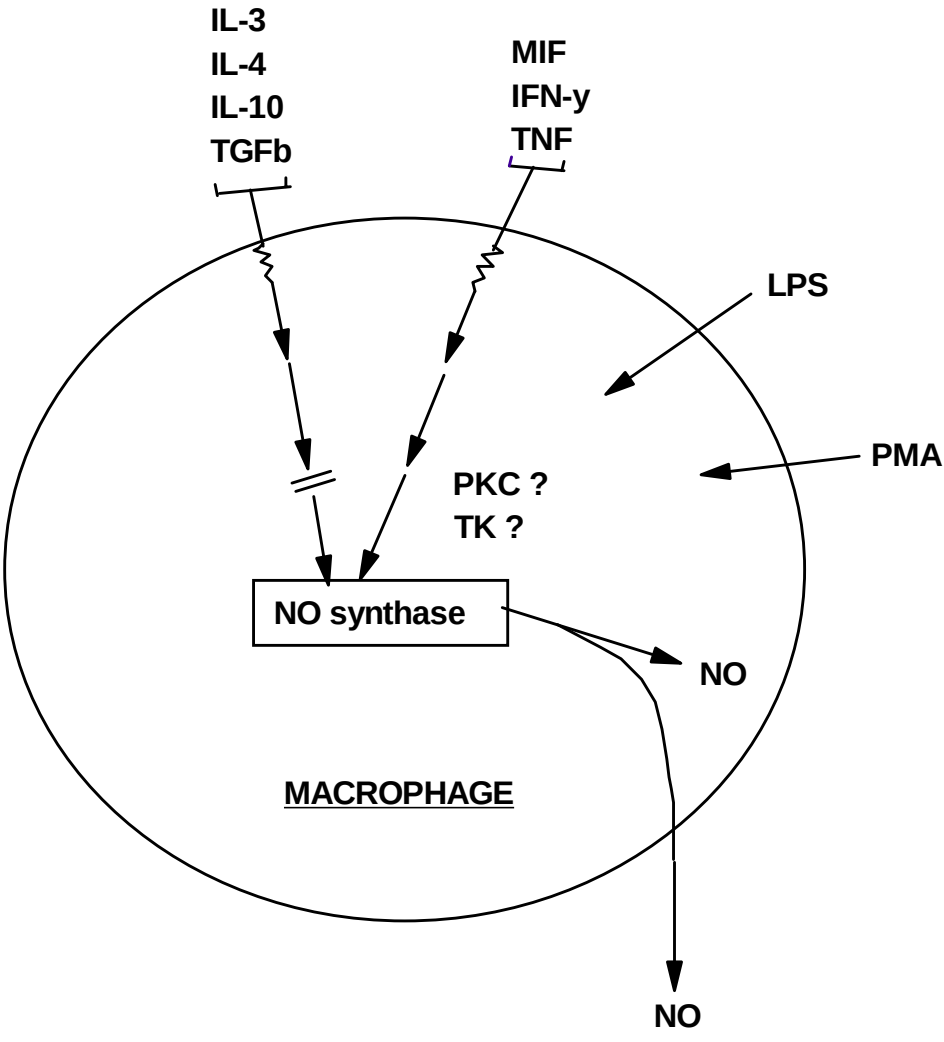

Figure 2. The involvement of cytokines in the regulation of nitric oxide production. Nitric oxide synthesis is stimulated by cytokines such as interferon-gamma and tumour necrosis factor and inhibited by cytokines such as IL4 and IL-5. After Liew and O'Donnell, 1993.

Novel vaccines should, therefore, take into account the immunogenicity of the epitope and the way it is recognized and processed, the pathways by which subsets of T cells are activated and the resulting cytokine profiles. So far, most information is available from studies on *L. major* but the principles derived from such studies can be applied to infections with *Plasmodium, Babesia, Theileria, Toxoplasma and Trypanosoma.* New approaches to vaccines might even overcome the inherent problems of antigenic variation in African trypanosomes and autoimmunity in Chagas disease. There is a number of ways in which this might be achieved but all depend on a detailed understanding (not just knowledge) of the whole pattern of events that occur when an animal is infected with a protozoan parasite. It should be possible, for example to drive an immune response towards protection and away from counter protection by

introducing a particular cytokine at the same time as a vaccine. This has been demonstrated experimentally in mice infected with *L. major in* which a disease enhancing antigen has been converted into a protective one by introducing tumour necrosis factor at the same time as the antigen (Liew *et al* ., 1991).

Vaccines of the future will have to be designed to take into account the cytokine profiles produced and it will no longer be acceptable simply to aim for a protective immune response without considering any possible adverse effects. It may be that protective vaccines that induce sterile immunity are unattainable and that what is necessary is to prevent disease rather than infection. This approach is being pioneered by Prof. Playfair and his colleagues for malaria but it may be appropriate for other infections also. For example, monoclonal antibodies directed against particular cytokines or their receptors could be used to ameliorate a potentially damaging response. Future progress will also require a willingness to accept mathematical modelling as a means of analysing and understanding the complex networks of cytokine interactions that occur during an infection; a start has already been made in this direction (Schweitzer and Anderson, 1992; Schweitzer *et al* ., 1993). A cytokine-based approach to developing new vaccines and control strategies will therefore require several changes of emphasis and thinking, but there is no easy route to the successful prevention of disease.

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Murine infection with *Leishmania major:* an experimental model for the study of interactions between intracellular pathogens and the immune systems of their hosts

*J.A. Louis, G. Del Guidice, K. Swihart, U. Fruth and P. Launois**

World Health Organization Immunology Research Training Centre
Institute of Biochemistry
University of Lausanne
1066 Epalinges, Switzerland

* on leave of absence from the Pasteur Institute

INTRODUCTION

Infections with *Leishmania* parasites, which live only in macrophages of mammalian hosts, represent a spectrum of diseases dependent upon the species of the parasite and the immune response of the host. Clinical manifestations of human cutaneous leishmaniasis can be reproduced in mice of different inbred strains following infection with *Leishmania* major Although the majority of inbred strains of mice (e.g. C57BL/6, CBA, C3H) are resistant to infection, a few strains, such as BALB/c, are susceptible to infection, develop severe cutaneous lesions and do not become immune to re-infection (Howard *et al.,* 1980).

There is no evidence that antibodies play a role in the elimination of the intracellular *Leishmania* spp. The clearance of these intracellular organisms requires the development of effective T cell-mediated immunity capable of activating infected macrophages to a microbicidal state.

$CD4^+$ T-CELL RESPONSES AND PATTERN OF DISEASE FOLLOWING EXPERIMENTAL INFECTION WITH *LEISHMANIA MAJOR*

Using the murine model of infection with *L. major,* resistance and susceptibility to infection was correlated with the expansion of $CD4^+$ T cells from unctionally distinct subsets (Heinzel *et al* ., 1991). Thus, during infection with *L. major,* susceptible BALB/c mice mount a polarized Th2-type response (e.g. an IL4, IL5, IL 1 0 producing $CD4^+$ T-cell response [Mosmann *et al.,* 19861]), whereas resistant C57BU6 or C3H mice develop mainly athl-type response (e.g. an IFNγ, IL2 producing $CD4^+$ T-cell response) (reviewed in Locksley and Scott, 1991). Furthermore, susceptible BALB/c mice rendered resistant by administration of anti-CD4 or anti-IL4 MAbs at the onset of infection mounted a predominantly

Th1-like response (Heinzel *et al* 1991). This concept of a strict correlation between virtual absence of Th l-type $CD4^+$ T-cell response and susceptibility to infection should be amended in the light of recent reports that IFNγ mRNA was detected in similar levels in lymph nodes draining lesions in strains of mice covering the entire range of susceptibility to *L. major (Morris et al* 1993a). Nonetheless, these studies did confirm that resistance to infection correlated with reduced amounts of IL4 mRNA and smaller numbers of cells producing IL4 (Morris *et al* ., 1993b).

The influence of L. major-specific Thl - and Th2-type $CD4^+$ T cells on pathogenesis has been assessed by studying the effect of adoptive transfer of differentiated T-cell lines into SCID mice. SCID mice reconstituted with Thl-like T-cell lines were able to restrict parasite growth, whereas mice reconstituted with Th2-like cell lines developed exacerbated disease (Holaday *et al.*, 1991). These results indicate that differentiated Thi and Th2 cells respectively are capable of mediating resistance and susceptibility to infection with *L. major*.

IFNγ is an important anti-L. *major* effector molecule because of its macrophage activating properties (reviewed in Liew and O'Donnell, 1993), while IL4 has been reported to block the activation of murine macrophages by IFNγ *in vitro (Liew et al.*, 1991). Similar observations have been made with human monocyte-derived macrophages infected with *L. amazonensis (Lehn et al.*, 1989; Ho, 1992). Evidence for a role of IL4 in susceptibility also derives from observations that resistant mice are rendered susceptible to infection by the expression of transgenic IL4 (Leal *et al.*, 1993). However, although unable to clear the infection, these mice exhibited lesions that were significantly smaller than those developing in genetically susceptible mice.

EVIDENCE THAT THE PROTECTIVE CAPACITY OF SOME *LEISHMANIA MAJOR* SPECIFIC $CD4^+$ THI T CELLS DEPENDS UPON THEIR SPECIFICITY

Some L. major-specific $CD4^+$ T-cell lines and clones, in spite of expressing a functional Thl-type phenotype, exacerbate disease in mice infected with *L. major (Muller et al* ., 1989). These parasite-specific Thl-type cells induced exacerbation in a dose-dependent manner when transferred into synagenic recipient mice, even when these were rendered T-cell and B-cell deficient before reconstitution (Titus *et al* ., 1991). Ovalbumin-specific Thl cells also exacerbated disease, but only if the cognate antigen, i.e. ovalbumin, was co-injected with the parasites. Following transfer into infected mice, these parasite-specific Thl cells migrate preferentially to the site of lesions. These results suggest that the major signal for exacerbation of disease by Thl-type $CD4^+$ T cells is their activation *in situ*. The observation that enhanced *L. major* growth in lesions of irradiated mice, which occurs as soon as four days after transfer of these Thl-like cells and simultaneous infection, is dependent on the administration of bone marrow cells as a source of circulating monocytes, indicates that this exacerbation of disease requires an interaction between Th l cells (or their products) and macrophages (Mendonea *et al* ., 1990).

Th1 -type $CD4^+$ cells capable of enhancing lesions were selected by *in vitro* restimulation with a lysate of promastigotes, the extracellular form of the parasite. In contrast, other parasite-specific Thl-like cells, recognizing only living parasites (Muller and Louis, 1989), protected normal mice against infectious challenge. These results highlighted key differences between Thl-type $CD4^+$ cells that recognize living parasites and those responding to dead parasite products in their effects on disease. The inability of non-proteetive parasite-specific Th1-type $CD4^+$ T cells to recognize their epitope on the surface of infected macrophages may result in their failure to focus the IFNγ on the infected cell and so restrict parasite growth (Locksley and Louis, 1992).

Two reasons might explain the failure of L. major-infected macrophages to present specific epitopes to $CD4^+$ T cells specific for antigen(s) present in a lysate of *L. major* promastigotes. The antigenic composition of the *L. major* amastigote intracellular form differs from that of the extracellular promastigote used to derive 'exacerbating' Th1-type $CD4^+$ T cells. In addition, even if similar antigens are present on both stages, their presentation to T cells by macrophages may be altered by the presence of living parasites in the compartment in which processing for the MHC-class II pathway of antigen presentation occurs.

The second possibility has been tested by studying the influence *L. major* inside macrophages on their antigen-presenting function. Bone marrow-derived macrophages infected with *L. major* have a greatly reduced ability to present non-parasite antigens to specific T cells. However, the ability of infected macrophages to present peptide epitopes to specific T-cell hybridomas was normal (Fruth *et al.,* 1993). Thus, the impaired ability of parasitized macrophages to present epitopes from erogenous protein antigens to T cells was not related to a lack of membrane MHC class II molecules for binding to newly formed peptides. Neither did this inhibition of presentation of protein antigen by parasitized macrophages result from a decrease either in the antigen uptake or its intracellular degradation. These data suggest that parasites in macrophages interfere with the intracellular loading of MHC class II molecules with antigenic peptides derived from exogenously added antigens. Interestingly, using a T-cell hybridoma specific for epitopes in a lysate of *L. major* promastigotes, it was shown that infected macrophages are profoundly affected in their ability to present exogenously added antigens of *L. major* to specific T cells (Louis *et al* ., 1993; Fruth *et al* ., 1993). These results could explain why 'exacerbating' Th1-type $CD4^+$ T cells specific for antigen(s) present in a lysate of *L. major* do not recognize their specific epitope at the surface of macrophages parasitized with *L. major.*

REGULATION OF $CD4^+$ T-CELL SUBSET DIFFERENTIATION DURING INFECTION WITH *LEISHMANIA MAJOR*

Differentiated Th1 - and Th2-type $CD4^+$ T cells arise from a common lineage (Rocken *et al* ., 1992; Seder *et al* ., 1992). The specificity of the T-cell receptor is not important in determining the pathway of $CD4^+$ T-cell precursor differentiation (Reiner *et al* ., 1993). Although it is becoming clear that several factors may influence the T-cell subset that predominates following antigenic stimulation, the precise understanding of the mechanisms involved in the

initiation of Thl or Th2 $CD4^+$ T-cell development from naive precursors remains an important and not fully resolved issue.

Results from *in vivo and in vitro* studies strongly suggest that cytokines present at the initiation of infection with *L. major* play a crucial role in the differentiation of naive $CD4^+$ T cells towards one or other functional phenotype. Administration of anti-IFNγ MAb at the time of parasite inoculation abrogated the natural resistance of C3H mice to infection (Belesovicetal., 1989). This treatment ablated the Thl-cell response normally seen during infection of these mice and promoted a Th2-type response (Scott, 1991). A role for IFNγ in driving the early $CD4^+$ T-cell response towards a Th l -type functional phenotype was also revealed by injecting recombinant IFNγ into susceptible BALB/c mice along with the infective inoculum; the cytokine profile was indeed altered towards a Th l -type response (Scott, 1991). The observation of an IFNγ response in lymph nodes draining the infection site of resistant mice as soon as 72 hours after infection supports the role of IFNγ in directing early $CD4^+$ Th1-type response. Natural killer (NK) cells have been shown to be the major source of IFNγ early during infection (Sharton and Scott, 1993). Similarly, the differentiation of adoptively transferred $CD4^+$ T cells towards a Th1 phenotype in infected SCID mice and the associated resistance of these mice to infection has been attributed to IFNγ produced by NK cells (Varkila *et al.*, 1993). However, IFNγ does not support a sustained Th1 response, since administration of IFNγ to susceptible BALB/c mice for up to six weeks after infection did not modify the outcome of disease (Sadick *et al* ., 1990). Furthermore, infection of BALB/c mice with parasites transfected with the murine IFNγ gene, led to the development of severe lesions and did not modify the polarized Th2-type response (Tobin *et al* ., 1993). These findings suggest that, in the murine model of infection with *L. major*, IFNγ alone is not sufficient either to promote a sustained differentiation of Th l cells in resistant mice or to impede Th2 cell maturation in susceptible mice. Recently, observations made in IFNγ-deficient C57B2/6 mice have revealed that these mice, in contrast to wild type control mice, develop a Th2-type response following infection with *L. major*, without evidence of a Thl-type response, supporting a role for IFNγ in the differentiation of Thl-type $CD4^+$ T cells (Wang *et al* ., 1994). In contrast, recent results showing that mice lacking the ligand binding chain of the Il.PNy receptor (IFNγR-/-) still mount a polarized IFNγ-producing $CD4^+$ T-cell response without evidence for the expansion of IL4-producing $CD4^+$ T cells indicate that IFNγ-mediated signals are not necessary for the differentiation of $CD4^+$ T-cell precursors towards a Th l phenotype (Swihart *et al* ., 1995). The reasons for this difference in the type of $CD4^+$ T-cell response triggered in IFNγR-/- and IFNγ-knockout mice following infection with *L. major* are not known. However, since the IFNγ and IFNγR-1- mice were derived on genetically different backgrounds, it is possible that the influence of IFNγ on the differentiation of T-cell subsets may vary in different mouse strains.

IL12 is a cytokine produced by macrophages and B cells (Trinchieri *et al* ., 1993) that stimulates the production of IFNγ by T and NK cells. Therefore, inasmuch as the early IFNγ-producing NK cell response observed in resistant mice has been implicated in the development of a polarized Thl response (Sharton and Scott, 1993), it was logical to consider that IL 12 plays a major

role in Th1 cell differentiation in the model of infection with *L. major* Administration of IL12 during the first week of infection with *L. major* cured BALB/c mice. This treatment also favoured a Thl-type response and prevented a deleterious Th2-type response (Heinzel *et al* ., 1993). Furthermore, treatment with anti-IL 12 polyclonal antibody, for 25 days from the time of infection significantly exacerbated disease in resistant mice (Sypeck *et al* ., 1993). These data show that IL12 plays an important role in the development of Th1-type response following infection with *L. major.*

Treatment of BALB/c mice with anti-IL4 MAb at the initiation of infection renders them resistant, inhibits the expansion of Th2 cells and leads to the development of a Th1 response (Sadick *et al.,* 1990; Tobin *et al,* 1993). Administration of IL4 to resistant mice at the time of parasite inoculation enhanced the development of a Th2-type $CD4^+$ response but this change from the normally observed pattern of response was only transient (Chatelain *et al,* 1992). Results recently obtained in our laboratory also strongly support the role for IL4 in the differentiation of *L. major* specific T-cell precursors towards a Th2 functional phenotype. $CD4^+$ T cells isolated from mice seven days after infection with *L. major* produced small amounts of IFNγ and IL4 upon specific restimulation *in vitro.* After a few cycles of specific restimulation, the amount of IFNγ produced by these $CD4^+$ T cells decreased progressively, whereas the release of IL4 increased steadily. After six cycles of restimulation, these cells produced almost exclusively IL4 and no IFNγ. Addition of soluble IL4 receptors (provided by M. Widmer, Immunex Corp., Seattle, USA) to the cultures led to the differentiation of T cells producing large amounts of IFNγ and almost no IL4 (manuscript in preparation). However, it was also reported that despite the administration of IL4 for three weeks following infection, resistant mice resolved their lesions (Sadick *et al* ., 1990). Thus, although IL4 might be required for the development of polarized Th2 response following infection with *L. major,* other factors might also be necessary.

CONCLUDING REMARKS

Infection of inbred mice with *L. major* remains one of the best experimental systems for the study of the complex interactions between intracellular pathogens and their hosts. Using this model system, basic research aimed at the identification of host factors that lead to or circumvent the development of an effective immune response has already greatly contributed to the demonstration of host-protective (Thl-type) and disease promoting (Th2-type) $CD4^+$ cell responses. The dramatic effects of these functionally distinct $CD4^+$ T cells on the disease processs has already been clearly related to the lymphokines that they produce.

This experimental model of infection provides a unique system for defining the rules that control maturation and activation of distinct $CD4^+$ T-cell subsets during antigenic stimulation *in vivo.* This not yet fully resolved issue has profound implications not only for the field of leishmaniasis, but also for a number of other diseases with an immunological basis.

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Inductive requirements of *Theileria parva*-specific cytotoxic T lymphocytes

E.L.N. Taracha and D.J. McKeever

International Laboratory for Research on Animal Diseases
P.O. Box 30709, Nairobi, Kenya

INTRODUCTION

There is strong evidence that cattle immunized against *Theileria parva* by infection and treatment clear the parasite through the deployment of class I MHC-restricted parasitespecific $CD8^+$ cytotoxic T lymphocytes (CTL) (McKeever *et al.*, 1994). The induction of these responses is crucial to the development of an improved vaccine for *T. parva* and work is in progress to identify parasite antigens that elicit them. However, the factors involved in the generation and maintenance of these responses *in vivo* are poorly understood. Conflicting data from murine and human systems suggest that $CD8^+$ CTL specific for certain antigens can be induced *in vivo and in vitro* with or without help from $CD4^+$ cells (Sprent *et al* ., 1986.. Buller *et al* ., 1987). Other requirements such as the nature of antigen-presenting cells (APC), their surface molecules and secreted factors have also been shown to be important (Huang *et al* ., 1994).

We have used highly purified (96-99%) populations of $CD4^+$ and $CD8^+$ T cells co-cultured with autologous lymphocytes transformed with the Muguga stock of *T. parva* (TpM) to examine the activation requirements of naive and immune $CD8^+$ T cells *in vitro.* We have observed that $CD4^+$ T cells and their secreted products are crucial to the induction of naive and parasite-specific bovine memory $CD8^+$ CTL (Table 1). Cultures of naive or immune $CD8^+$ T cells with TpM in the presence of immune $CD4^+$ T cells generated $CD8^+$ CTL activity comparable to that of unfractionated cells. In addition, specific stimulation of $CD4^+$ T cells primed with an unrelated antigen, the variable surface glycoprotein (VSG) *of Trypanosoma brucei*, in similar co-cultures generated help for the activation of parasite-specific $CD8^+$ CTL from immune but not naive $CD8^+$ T cells. This activity was higher when antigen-pulsed and fixed monocytes presented the VSG (four-cell cluster) than when the parasitized cell line was used as APC (three-cell cluster).

To characterize helper signals delivered to $CD8^+$ T cells in these experiments, a supernatant of T-cell growth factors (TCGF) derived from Concanavalin A-stimulated lymphocytes or recombinant bovine interleukin-2 (rBoIL-2) were added to co-cultures of naive or immune $CD8^+$ T cells with TpM. Significant

Table 1. Activation of Theileria-immune and naive CDS^+ T cells is dependent on help from antigen-specific $CD4^+$ T cells.

Culture components	Killing activity[2]
iPBM[1] + TpM	+++
nPBM + TpM	+
iCD8 + TpM	+
iCD8 + iCD4 + TpM	+++
iCD8 + nCD4 + TpM	+
nCD8 + iCD4 + TpM	+++
nCD8 + iCD4 + TpM	+

1. i = immune, n = naïve, M = periipheral blood monocytes.
2. + = <10%, ++ = 10-25%, +++ = >25-55%.

Table 2. Antigen-primed $CD4^+$ T cells need not be *Theileria-specific* in order to provide helper function to parasite-specific CTL.

Culture components	Killing activity[2]
VSG-specific CD4 + iCD8[1] + TpM + VSG	+++
VSG-specific CD4 + iCD8 + TpM + MO + VSG	+++
VSG-specific CD4 + nCD8 + TpM + VSG	++
VSG-specific CD4 + nCD8 + TpM + MO + VSG	+

1. i = immune, n = naïve, M = periipheral blood monocytes.
2. + = <10%, ++ = 10-25%, +++ = >25-55%.

Table 3. Role of contact signals and soluble factors from antigen-specific $CD4^+$ T cells in the provision of helper function to immune and naive $CD8^+$ T cells.

Inner chamber	Outer chamber	Killing activity[2]
iCD4[1] + TpM	iCD8 + TpM	+++
iCD4 + TpM	nCD8 + TpM	+
None	iCD41 + nCD8 + TpM	+++
TCGF + medium	iCD8 + TpM	+++
TCGF + medium	nCD8 + TpM	+
rBoIL-2 + medium	iCD8 + TpM	+++
rBoIL-2 + medium	nCD8 + TpM	+

1. i = immune, n = naïve, M = periipheral blood monocytes.
2. + = <10%, ++ = 10-25%, +++ = >25-55%.

CTL activity, comparable to that obtained with immune $CD8^+$ T cells stimulated by TpM in the presence of immune $CD4^+$ T cells, was detected in cultures containing primed $CD8^+$ T cells but not in those containing naive cells.

To determine whether contact between parasite-specific CD4+ T cells and CD8+ T cells is an essential requirement for helper function, additional experiments were performed using two-chamber culture plates incorporating semi-porous membranes. TpM were co-cultured with naive or immune CD8+ T cells in the outer chamber and with immune CD4+ T cells in the inner chamber. Cytotoxic activity was observed in cultures containing primed CD8+ T cells but was undetectable in those containing naive CD8+ T cells. This observation suggests that while soluble factors derived from activated CD4+ T helper cells are sufficient for the activation of memory CTL, naive CD8+ T cells require actual cell contact. Whether this signifies the involvement of receptor-ligand interactions or is a reflection of a requirement for defined local cytokine concentrations is not clear.

SUMMARY

It is evident from these results that Theileria-immune and naive CD8+ T cells require help from activated antigen-specific CD4+ T cells to generate parasite-specific cytotoxic activity. The CD4+ T cells need not be Theileria-specific, although this bystander effect is operative for primed CD8+ T cells only. While TCGF or rBoIL-2 can replace the requirement of primed CD8+ T cells for CD4+ T cell help, these factors cannot induce CTL activity in naive CD8+ T cells cultured with autologous parasitized lymphoblasts. The results further suggest that contact signals from immune CD4+ T cells are important for the specific activation of naive CD8+ cells.

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A role for γδ T cells in theileriosis

C. Daubenberger

International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi, Kenya

T cells bearing the γδ antigen receptor can constitute up to 50% of T cells in peripheral blood and lymphoid organs of calves below six months of age. This observation prompted us to investigate the involvement of γδ T cells in the immune response of young calves against *Theileria parva* (Muguga).

A limiting dilution analysis of γδ T cells sorted from peripheral blood of one calf was conducted to examine the frequency of proliferating γδ T cells changes during the course of a primary infection. Three different stimulator cell populations, namely autologous Con A blasts, autologous heat-stressed (HS) Con A blasts and autologous *T. parva*-infected cell lines (TpM) were used in the assay. This analysis revealed that *T. parva-specific* γδ T cells were present at measurable frequencies prior to immunization, while those responding to Con A blasts or HS Con A blasts were not detectable. Frequencies of proliferating γδ T cells responding to HS Con A blasts and TpM increased dramatically during immunization and dropped after clearance of the infection.

To investigate the basis of the reactivity of bovine γδ T cells for autologous infected cell lines, a panel of γδ T-cell clones has been generated. The phenotype of these T-cell clones is $CD3^+$, $CD2^+$, CD4-, $GB21A^+$ and $CC15^+$, and a proportion express the CD8 specificity. These T-cell clones proliferate specifically in the presence of autologous and allogeneic TpM. The proliferative response can be increased in some clones by heat shocking the TpM. This reactivity can be blocked by monoclonal antibodies against the bovine γδ T-cell receptor (GB21A) and CD2 (IL-A 43). Some γδ T-cell clones can be activated by fixed autologous TpM, although to a lower degree. Both IL-2 and IL-4 are required for proliferation of these clones in the presence of TpM.

Since it has been established that a proportion of γδ T cells in mouse and human are activated by stress proteins, we examined the possibility that *T. parva*-specific γδ T cells recognize stress proteins by characterizing the expression of the hsp70 family of stress proteins in *T. parva*-infected cell lines. A rat polyclonal sera has been raised against the *T. parva*-derived hsp70 and gives rise to intense punctate staining of the schizont cytoplasm in immunofluorescence analysis of infected cells; staining of the host cell cytosol is variable and of lower intensity. The latter observation is probably the result of cross-reactive epitopes located on bovine and parasitic stress proteins, since hsp70 proteins are known to be highly conserved. Staining of the schizont is markedly intensified by prior incubation of the infected cells at 42 'C for 3 h. Host cytoplasm staining is unaffected by this treatment.

In vivo depletion of T-cell subsets in cattle

J.-P. Scheerlinck and J. Naessens

International Laboratory for Research on Animal Diseases
P.O. Box 30709, Nairobi, Kenya

INTRODUCTION

Techniques for *in vivo* depletion of lymphocytes using monoclonal antibodies have been well documented for murine systems. This model has played an important role in analysing the involvement of various lymphocyte subsets in several immune mechanisms. In mice 600 μg of Ab are generally injected intraperitoneally once a week. In order to study bovine specific immune mechanisms we have attempted to adapt this technology to cattle.

RESULTS AND DISCUSSION

Previous studies indicated that intravenous inoculation of cattle with MAb specific for bovine T-cell markers resulted in depletion of the appropriate T-cell subsets in peripheral blood (Howard *et al.*, 1989, 1992). However, it was observed that, with this dose of MAb, T-cell depletion of other lymphoid organs was not complete. Furthermore, intravenous inoculation of these quantities of MAb was occasionally accompanied by shock-like symptoms. Since it has been observed that the depletion blood can be complete in less than two hours, the latter may be related to massive agglutination and lysis of the target cell population. We modified the inoculation regime by administering only a small dose of MAb in the first injection. In studies that focused on $CD4^+$ and $CD8^+$ T cells, reconstitution of the blood with the depleted population was observed overnight, presumably the result of migration of T cells from lymphoid organs. The treatment was continued by injection of small amounts of antibodies in the morning followed by a high dose in the afternoon (Table 1). Using this protocol the majority of the animals did not show adverse reactions, although some did show transient mild reactions. Complete depletion of peripheral blood was achieved and animals could be maintained on this regime for up to four weeks. After this period $CD8^+$ T cells reappeared in the blood even under continuous antibody treatment. This is most likely due to the formation of anti-mouse Ig antibodies in cattle. Indeed, bovine anti-mouse-Ig antibodies can be detected in serum of treated animals as early as one week after the first injection. These antibodies may be involved in clearing the active mouse monoclonal antibodies but may also contribute to aggregation of T cells.

We have performed an immunohistological analysis of the extent of depletion in lymphoid tissues in one animal sacrificed after complete depletion of $CD8^+$ T cells in peripheral blood. Considerable variation in the extent of the depletion

was observed in different immune compartments. Using FACS analysis of cell suspensions, extensive $CD8^+$ T-cell depletion was observed in the blood and lymph node but not in spleen. By immunohistology, $CD8^+$ positive cells were shown to be present in the lymph node, spleen and thymus.

Table 1. *In vivo* depletion of bovine T-cell subsets using monoclonal antibodies.

Day of inoculation	Morning	Afternoon
Day 1	0.5 mg	0.5 mg
Day 2	0.5 mg	2.0 mg
Day 3	2.0 mg	20.0 mg
Day 4	2.5 mg	20.0 mg

To investigate the functional significance of the remaining $CD8^+$ T cells, an *in vitro* restimulation assay was performed on different tissues, based on reactivity to *T. parva, to* which the animal was immune. Lymphocytes were co-cultured with irradiated *T. parva*infected stimulator cells for one week and analysed for their capacity to kill parasitized target cells (Goddeeris *et al* ., 1986). It was observed that lytic activity could be detected in cells derived from lymph node, spleen and even peripheral blood (data not shown). Since only very small numbers of $CD8^+$ T cells were detectable in these populations by FACS analysis prior to stimulation, it is clear that the *in vitro* restimulation assay is much more sensitive. This might be related to the expected expansion of $CD8^+$ cells during the one week *in vitro* culture in the presence of *T. parva*-infected cells. Moreover, these experiments show that cell suspensions such as those that might be collected in aspirated lymph node biopsies are not representative of total lymph node.

In an attempt to target antibodies more directly to the lymph node we administered MAb to one cow subcutaneously. At the first inoculation large amounts of antibodies (5 mg) were very well tolerated and no side effects were recorded. Three days after injection of anti-$CD8^+$ antibodies very good depletion was observed in the blood. However, depletion in both blood and lymph node were not complete by day 10. Interestingly, no chancre formation was observed when this animal was infected with *T. congolense* through bites of infected tsetse flies. Since the chancre formation is mainly the result of infiltrating lymphocytes, this may suggest that the level of T-cell depletion in the skin can influence the response of cattle to the bite of an infected tsetse fly (Table 2).

To determine whether T-cell memory is affected by cell depletion, we examined the capacity of cattle that had been allowed to recover blood T-cell numbers following complete depletion. We reasoned that if all T cells were depleted, a memory response would not be present in the reconstituting population. Foot and Mouth Disease virus (FMDV) antigen constituted an ideal system for this investigation since all of our animals had been immunized against this virus. Animals were treated with MAb specific for CD8 or both

CD4 and CD8 for one week and allowed to reconstitute the depleted population(s). After 36 days of reconstitution proliferative activity against FMDV antigen was measured in peripheral blood of (i) normal, (ii) $CD8^+$ T cell-depleted and (iii) $CD4^+$ and $CD8^+$ T cell-depleted animals (Table 3). In two animals depleted of both $CD4^+$ and $CD8^+$ T cells, no FMDV-specific T-cell proliferation could be observed (Table 3). The control animals and the $CD8^+$ T-cell depleted animals showed normal T-cell proliferation.

Table 2. Chancre formation in a $CD8^+$ T cell-depleted animal on day 10 after feeding of infected tsetse fly bites. Average skin thickness (mm) around five bite sites.

Normal skin Chancres	
Control	$CDS^+/CD4^+$ T cell-depleted
8	8
14	11
16	11
18	11
18	11
18	12

Spontaneous recovery of specific T-cell activity in one of the calves depleted of both $CD4^+$ and $CD8^+$ T cells might have been the result of residual vaccine antigen present during reconstitution. This antigen could prime naive T cells leaving the thymus. However, it cannot be excluded that memory $CD4^+$ T cells escaped the depletion and only entered the peripheral circulation in the later stages of reconstitution. Both animals recovered normal proliferative responses after re-immunization with the antigen of interest, suggesting that a normal immune response is possible in animals that have recovered from immune cell depletion, and that only T-cell memory is affected by this treatment. Taken together these results provide preliminary indications that *in vivo* depletion of $CD4^+$ T-cell memory in peripheral blood is possible.

Table 3. FMDV-specific proliferation of PBM from animals depleted for specific T-cell subsets and allowed to reconstitute normal cell numbers. Figures represent average counts of duplicate wells adjusted for background.

Days of reconstitution	Intact		Depleted $CD4^+$ and $CD8^+$ T cells		Depleted $CD8^+$ T cells	
	BK 66	BK 68	BK 69	BK 70	BK 71	BK 72
36	34706	6528	78	46	30224	2911
43	24226	14026	265	868	59671	2438
59	4987	2994	0	4242	11695	1493
69	9056	9342	0	5485	76599	3592
78			IMMUNIZATION WITH FMDV			
85	2188	4700	7931	20317	25897	1395
91	28196	14419	37821	31291	62137	11472

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Summary of discussion

Chairperson: *J. Naessens*
Rapporteurs: *N. McHugh and C. Wells*

The session opened with an overview by F. Cox of the roles of cytokines and T-cell subsets in the immunity and immunopathology of protozoan parasitic diseases. J. Louis then described the current perception of immunity of mice to *Leishmania major* infection, which is the most extensively studied model of Thl and 712 subsets and the cytokines that they produce. Some observations on the inductive requirements of bovine $CD8^+$ cytotoxic T cells were presented by E. Taracha and preliminary data on the involvement of γδ T cells in *T. parva* and trypanosome infections of cattle were discussed by C. Daubenberger and M. Sileghem respectively. In the final presentation of the session, J-P Scheerlinck described attempts to deplete bovine T-cell subsets *in vivo* by treatment with lineage-specific monoclonal antibodies.

In the discussion that followed it was observed that in spite of the clear cut dichotomy between Thl and Th2 $CD4^+$ T-cell responses in protection of mice against *Leishmania major,* no such distinction exists in many diseases. Indeed in leprosy the best prognosis is for those patients with a mixed Thl/Th2 response. It was suggested that, since immune responses occur within discrete microenvironments, the generation of a specific Thl response to an antigenic challenge need not preclude the development of a Th2 response in another location. There was some debate on the importance of both antigen type and route of administration in the generation of Th1 and Th2 responses. The initial antigen presentation event was considered to be a major factor in determining the direction of the response, although it was observed that individual peptide antigens can elicit distinct responses when administered through different routes of inoculation. The significance of the latter finding was questioned on the basis that the peptides involved were not targets for protective immune responses.

There was some discussion on the ability of *Leishmania major* to access the MHC class I antigen processing pathway, since the parasite survives within a phagolysosome. It was suggested that the mechanisms responsible for translocating peptides from the cytosol to the phagolysosome might be reversible and allow peptides or proteins to exit to the cytosol and become available for MHC class I processing.

The mechanism by which *T. parva* peptides might be presented to specific $CD4^+$ T cells to elicit help for naive and primed $CD8^+$ T cells was discussed. The possibility was raised that $CD8^+$ T cells may be capable of presenting antigen in association with class II MHC, and so elicit their own help. It was pointed out that activated T cells in cattle express class II MHC and that activated bovine $CD8^+$ T cells have been shown to present trypanosomal variable surface glycoprotein to specific $CD4^+$ T-cell clones. However, it was

observed that the basis of class II MHC-associated antigen presentation in the *T. parva* system was still under investigation.

Possible mechanisms whereby γδ T cells recognize antigen were discussed and it was observed that in man heat shock proteins (hsp) have been shown to be presented to γδ T cells by non-classical class I MHC molecules. It was suggested that hsp antigens may be involved in activation of γδ T cells in both ECF and trypanosomiasis. It was however stressed that the study of bovine γδ T cells is at a preliminary stage and that their precise function and mechanisms responsible for their control remain to be elucidated.

A lengthy discussion focused on the *in vivo* depletion of bovine T-cell subsets using monoclonal antibodies. Two major concerns that arose were the extent of depletion that was possible in cattle and, given the complicated nature of ECF and trypanosomiasis, whether the results of depletion experiments in these systems could be interpreted accurately. The extent of depletion was considered to be critical since failure to remove precursor cell populations completely could compromise the interpretation of results. Suggestions were made on how greater levels of depletion might be obtained; osmotic pumps or hybridoma cell lines encapsulated in sodium arsenate and poly-L-lysine to allow persistent levels of antibody might improve the extent of depletion. However, it was pointed out that the possibility of hypersensitivity and the accumulation of large amounts of host antibodies to mouse Ig would still be possible sources of complication. Although it was generally agreed that good depletion data would complement adoptive transfer studies, it was felt that the complex nature of *T. parva* infections, where T-cell depletion would be removing both parasitized host cells and immune effector populations, would seriously complicate the interpretation of results. However, it was suggested that the technique might be applicable in the study of levels of T-dependent antibody production in Boran and N'Dama cattle undergoing infection with trypanosomes, where even partial depletion of target cell populations would be sufficient to produce a measurable result.

SELECTION OF PROTECTIVE RESPONSES THROUGH IMMUNIZATION STRATEGIES

The role of immune responses to selected viral antigens in immunity to infection and enhanced disease

G. Taylor and W.L. Morrison

AFRC Institute for Animal Health
Compton, Nr Newbury
Berkshire RG16 0NN, UK

INTRODUCTION

Pathogenic organisms are often capable of stimulating a range of humoral and cell-mediated immune responses, only some of which are relevant in mediating recovery from infection and subsequent immunity to challenge. Indeed, in some infections, components of the immune response have been shown to contribute to disease, and the balance of the immune responses determines whether the outcome of infection is recovery or enhanced disease (Scott and Kauffmann, 1991). This is particularly well-documented for a number of parasitic infections where the outcome is determined by the profile of cytokines produced by $CD4^+$ T cells (Scott *et al.*, 1989). Recently, evidence has emerged that similar phenomena operate in viral infections (Clerici and Shearer, 1993; Alwan and Oppenshaw, 1993). In considering approaches to vaccination against such organisms, the identification of both the beneficial and harmful elements of the immune response and definition of their antigenic specificities are necessary steps toward choice of antigens and appropriate antigen delivery systems. Because of the relatively simple antigenic structure of viruses and the availability of detailed information on genome sequences, the immune responses elicited by viruses are particularly amenable to detailed dissection. This presentation will summarize current information on the nature and antigenic specificity of immune responses to respiratory syncytial virus (RSV) and consider their role in control of infection and in the pathogenesis of pulmonary lesions.

RESPIRATORY SYNCYTIAL VIRUS

Bovine RSV infects the epithelium of upper and lower respiratory tract and is an important cause of pneumonia in housed calves (Stott and Taylor, 1985). Outbreaks occur annually during the winter months with greatest mortality being observed during the first six months of life, when maternal antibody is often still present. Reinfection is common although the severity of disease usually decreases with subsequent infections. RSV is a pneumovirus of the Paramyxoviridae family and contains a negative sense single-stranded RNA

encoding ten proteins seven of which are incorporated in the virion. The attachment (G) and fusion (F) glycoproteins are expressed on the outer aspect of the viral envelope. The bovine virus is structurally and antigenically related to human RSV and the epidemiology and pathogenesis of the diseases they cause are very similar. A number of vaccines of variable efficacy have been produced for human and bovine RSV. A vaccine consisting of glutaraldehyde-fixed infected cells has proved to be particularly effective in calves (Stott *et al* ., 1984; Howard *et al* ., 1987). However, with some of the vaccines there have been reports of enhanced pathology following natural challenge of vaccinated individuals (Kimman *et al* ., 1989; Kim *et al* ., 1969). This was a particular problem with a human vaccine that employed formalin-fixed virus. These observations highlight the need for a clearer understanding of the mechanisms of immunity against RSY.

In contrast to bovine RSV, which is not infectious for mice, human RSV readily infects mice and causes pneumonia (Prince *et al* ., 1979; Taylor *et al* ., 1984a). While the murine model has proved to be a valuable system for exploring the nature and specificity of immune responses induced by the virus, there is evidence that the relative contribution of the components of the immune response to immunity and enhanced pathology differ from that in the natural hosts. The information presented herein is drawn from studies of the human virus in the murine model and the bovine virus in cattle.

IMMUNOGENICITY OF VIRAL GLYCOPROTEINS

Studies of the immunogenicity of human RSV proteins have been carried out in mice using biochemically purified viral proteins (Routledge *et al* ., 1988) or recombinant vaccinia viruses expressing individual proteins (Stott and Taylor, 1989). In both instances, only the G and F glycoproteins stimulated significant protection in the lung against RSV challenge; in mice immunized with these recombinant vaccinia viruses, titres of virus in the lungs following challenge were reduced by more than $\log_{10}2.5$ plaque-forming units compared with controls. Both glycoproteins induced neutralizing antibodies. Although vaccinated mice were protected against RSV infection, they developed more severe lung lesions after RSV challenge than control animals (Stott *et al* ., 1987; Openshaw *et al* ., 1992).

Preliminary experiments in calves indicate that immunization with recombinant vaccinia viruses expressing the F, G or N proteins result in reduced levels of virus replication in the lung. In contrast to the findings in mice, there was a marked reduction in the extent of lung lesions in vaccinated calves following RSV challenge compared with controls.

PROTECTIVE PROPERTIES OF GLYCOPROTEIN-SPECIFIC ANTIBODIES

The role of antibodies in immunity has been studied by examining the protective activity of monoclonal antibodies (MAbs) specific for RSV proteins. The MAbs comprised a panel of reagents raised in mice against human RSV and 12 bovine MAbs, all specific for the F glycoprotein, produced by heterohybridomas generated from cattle immunized with bovine RSV. The MAbs were administered either intraperitoneally or intravenously to mice 24 h prior to intranasal challenge with human RSV. Protection, as judged by reduction in virus growth, was achieved only with MAbs specific for the F and G glycoproteins (Taylor *et al .*, 1984b; Kennedy *et al .*, 1988). Not all MAbs specific for these antigens were protective and the level of protection varied for different antibodies. When tested against a number of different strains of both human and bovine RSV, the protective F-specific MAbs were cross-reactive, recognizing all the human and bovine viruses studied. In contrast, G-specific MAbs were either strain-specific or specific for the subgroup of human or bovine RSY When the properties of protective and non-protective MAb specific for the F protein were compared there was no correlation between protection and the antibody isotype, the capacity to facilitate complement-mediated lysis or *in vitro* neutralizing activity (although all protective antibodies neutralized). However, a consistent correlation was found between the ability of the antibodies to inhibit fusion and syncytia formation by infected cells and protective activity (Taylor *et al .*, 1984b, 1992). That the protective activity of these MAbs was independent of their Fc domains was demonstrated by the finding that Fab fragments of the MAb were able to mediate protection.

Protective MAbs specific for the F glycoprotein were also tested for therapeutic activity by administering them to mice four days after infection with RSV, when high titres of virus are found in the lungs. Somewhat surprisingly, this treatment was found to reduce virus titres in the lungs to undetectable levels within 24 hours and again was independent of the Fc domain. A similar result was obtained in athymic nude mice indicating that the effect did not depend on primed T cells. Furthermore, virus did not reappear in the lungs of passively immunized athymic mice, even when the antibody had declined below levels previously shown to be protective. In contrast, administration of protective G-specific MAb to infected nude mice resulted in a more gradual decline in virus titres. The results of these studies indicate that antibodies specific for the F glycoprotein exert their protective effect directly as a result of binding to functional sites on the molecule. Presumably they are capable of preventing spread of virus from cell to cell, as well as clearing free virus. Whether the antibodies have additional effects on infected cells that result in inhibition of viral growth has yet to be investigated.

Since the F-specific MAbs reacted with both human and bovine RSV it has been possible to examine the protective activity of these antibodies in calves. Intra-tracheal administration of fusion-inhibiting bovine MAbs that were protective against RSV infection in mice, 24 h prior to challenge with bovine RSV, resulted in significant reduction in virus replication in the lung and a reduction in the severity of pneumonic lesions, indicating that these antibodies are also protective in cattle.

These studies have shown that certain antibodies to the F protein of RSV are highly effective both at preventing and clearing RSV infection. Furthermore,

antibody appears to either protect against lung lesions or have no effect on their development.

A combination of techniques has identified two sites on the F glycoprotein recognized by protective antibodies (Taylor *et al* ., 1992). By sequence analysis of the F protein gene from antibody-escape mutants selected with the protective MAb, these areas have been located to amino acid residues 255-275 and the region around amino acid 429 of the F protein (Arbiza *et al* ., 1992). These findings indicate that there are two immunodominant sites on the F protein recognized by protective antibodies. It is of note that the murine and bovine MAb reacted with the same sites on the molecule.

T-CELL RESPONSES IN MICE

Antigen-specific T-cell proliferative responses and MHC-restricted cytotoxic T cells have been demonstrated in mice infected with RSV (Taylor *et al* ., 1985; Openshaw *et al* ., 1988, 1990). A range of cytokines, including IL-2, IL-4 and IL-5, are produced by T cells from previously infected mice after restimulation with RSV *in vitro* (Alwan and Openshaw, 1993). Similar studies conducted in mice immunized with recombinant vaccinia viruses expressing the F or G glycoproteins have revealed a marked difference in the T-cell responses stimulated by the two antigens (Alwan and Openshaw, 1993). The F glycoprotein induced readily detectable CTL, abundant IL-2 production and low levels of IL-4 and IL-5. In contrast, the G glycoprotein induced a response characteristic of Th2 cells, namely the absence of detectable CTL and production of IL-4 and IL-5 but little if any IL-2. Similarly, F-specific cell lines, maintained by repeated stimulation with RSV, showed a Th1 pattern of cytokines whereas G-specific lines had a Th2 pattern of cytokine production (Alwan *et al* ., 1993). Although mice recover from primary infection with RSV or infection with vaccinia recombinants expressing the F or G glycoproteins control virus replication following challenge with RSV, such animals develop enhanced lung pathology compared with animals undergoing primary infection (Stott *et al* ., 1987; Openshaw *et al* ., 1992). The lung lesions are particularly pronounced in mice immunized with the G glycoprotein and include infiltrates of eosinophils, a feature associated with IL-5 production.

These findings provide important clues to the basis of the enhanced pathology that is sometimes observed after natural human and bovine infections of vaccinated animals. In particular, they suggest a role for Th2 T-cell responses. However, the observation of pathological lesions following secondary infection of mice with RSV is not a feature of experimental challenge infections in cattle and highlights the shortcomings of the murine model.

THE ROLE OF T-CELL RESPONSES IN RECOVERY FROM INFECTION IN CATTLE

The precise nature of the T-cell responses induced by RSV in cattle has not yet been investigated. However, the role of T-cell subsets in recovery from primary infections has been examined by monitoring the course of infection in calves

transiently depleted of T-cell subpopulations. Depletion of the $CD4^+$ or $CD8^+$ subsets of T cells was achieved by intravenous administration of specific MAbs. Although depletion of $CD4^+$ T cells resulted in a delay in the onset of RSV-specific antibody production, the course of infection was similar to that in untreated controls. By contrast, animals depleted of $CD8^+$ T cells exhibited a significant delay in clearance of virus from nasal secretions and the lungs. Thus, despite the evidence that antibodies play a prominent role in control of RSV the results of this study indicate that $CD8^+$ T cells are of critical importance in recovery from infection. The apparent failure of antibody produced in CD8-depleted calves to clear the virus perhaps suggests that the primary antibody response may be qualitatively defective These findings contrast with those in mice depleted of T-cell subsets, where failure to clear RSV only occurred in mice depleted of both $CD4^+$ and $CD8^+$ T cells (Graham *et al* ., 199 1). Current studies are aimed at defining the nature and antigenic-specificity of the $CD8^+$ T-cell response.

In summary, these studies indicate that the F protein is the major protective antigen of RSV, providing protection against different strains of virus. Fusion-inhibiting antibody is highly protective, although, at least in cattle, $CD8^+$ T-cell responses appear to be essential for recovery from primary infections. Furthermore, in contrast with the failure of antibody to potentiate disease, there is evidence that Th2 responses may contribute to the pathology of RSV disease.

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Experiences with immunization against *Theileria parva* using p67, a sporozoite surface antigen

A.J. Musoke, D.J. McKeever, S.P. Morzaria, C. Nkonge and V. Nene

International Laboratory for Research on Animal Diseases
P.O. Box 30709, Nairobi, Kenya

INTRODUCTION

Theileria parva, a protozoan parasite of cattle, causes a disease called East Coast fever (ECF) (reviewed by Irvin and Morrison, 1987). The disease is of economic importance because it causes high morbidity and mortality and limits the development of the cattle industry (Mukhebi *et al.,* 1991). Recent estimates have shown that 20 million cattle are at risk in eastern, central and southern Africa and indicate that in 1989 alone almost US$ 168 million were lost due to deaths and debilitation (Mukhebi *et al.,* 1991). The parasite is transmitted by the brown ear tick, *Rhipicephalus appendiculatus.* lie infective stage for cattle, the sporozoite, develops in the salivary glands of the tick vector and it is introduced into the mammalian host during tick feeding. Sporozoites are released into the host where they enter lymphocytes by receptor mediated endocytosis (Fawcett *et al.,* 1982; Shaw et al., 1991). $CD4^+$ and $CD8^+$ T-lymphocytes are the major target cells although B-Iymphocytes and y8 T cells can also be infected (Baldwin *et al.,* 1988). Within lymphocytes, sporozoites differentiate into the pathogenic schizont stage, an event that is associated with the transformation and clonal expansion of the parasitized cells. Schizonts undergo merogony to produce merozoites which are released upon rupture of the host cells. Merozoites invade erythrocytes where they differentiate into the piroplasm stage, the infective stage to ticks.

East Coast fever is characterized by pyrexia, generalized lymphadenopathy and severe leucopaenia and is usually fatal (Irvin and Mwamachi, 1983). However, animals that recover spontaneously or due to drug intervention are resistant to re-infection with the same stock of parasite (Radley, 1981), an indication that vaccination against the disease is possible.

The only available method for immunization against ECF is by infection with live sporozoites and simultaneous treatment with a long acting oxytetracycline (Radley, 1981). The method is not yet widely used because the immunity engendered is parasite strainspecific and because of cold chain and delivery problems related to poor infrastructure in ECF endemic areas. In addition, there are risks of transmitting other tick-borne pathogens. A subunit vaccine is therefore an attractive alternative for the control of ECF.

MECHANISMS OF PROTECTION AGAINST EAST COAST FEVER

The infection-and-treatment method of immunization gives rise to a transient parasitosis which is followed by the appearance of genetically restricted cellular responses directed against the schizont-infected cells. It is believed that this response is the major protective mechanism in immune cattle due to the observation that it appears in animals at the time of remission of the infection (Eugui and Emery, 1989) and that the immunity can be transferred using thoracic duct lymphocytes (Emery, 198 1). The T cells which are responsible for this activity have been cloned and shown to belong to $CD8^+$ subset of T-lymphocytes (Goddeeris *et al.,* 1986). The role played by these cells has recently been confirmed using transfer experiments which have clearly demonstrated that $CD8^+$ T cells can confer protection against a lethal challenge with *T. parva* sporozoites (McKeever *et al.,* 1994). However, recent evidence suggests that immune mechanisms directed against the sporozoite may also be protective. Sera from cattle in endemic areas or those immunized repeatedly with sporozoite lysates can neutralize the infectivity of sporozoites *in vitro* (Musoke *et al.,* 1984). Similar results have been obtained using monoclonal antibodies (MAbs) raised against surface antigen of *T. parva* sporozoites (Musoke *et al,* 1984; Dobbelaere *et al.,* 1984). In addition sera from animals immunized with one parasite stock are capable of neutralizing sporozoites from another stock (Musoke *et al.,* 1984). These sera and MAbs recognize a 67 kilodalton (kDa) stage-specific antigen (p67) in immunoblots (Nene *et al.,* 1992). Immunogold labelling studies using MAbs and immune sera have confirmed that p67 antigen is located on the surface of the sporozoites (lams et *al.,* 1990; Musoke *et al.,* 1993).

Further analysis with MAbs indicated that at least two B-cell epitopes are present on p67 and that these are conserved among the sporozoites of different parasite stocks (Musoke *et al.,* 1984). A vaccine based on p67 could therefore circumvent the stock specificity of the responses engendered by the infection-and-treatment method of immunization. Since the severity of the disease is dose-dependent (Jarret *et al.,* 1969), such a vaccine could still be effective even if only partial neutralization was achieved.

CLONING AND EXPRESSION OF P67

The isolation and characterization of the gene encoding p67 has been described previously (Nene *et al.,* 1992). The single copy gene, containing an intron of 29 base pairs, is transcribed only in the sporozoites, and encodes 709 amino acid residues. The predicted amino acid sequence incorporates a signal sequence at the N-terminus, seven sites for N-linked glycosylation and a hydrophobic C-terminus. The gene is present in all stocks of *T. parva* and has a high degree of homology with the gene encoding SPAG1, a *T.annulata* sporozoite surface antigen (Nene *et al.,* 1992). The primary amino acid sequences suggest that p67 is a membrane protein with a predicted molecular mass of 75 kDa. The protein does not contain multiple short amino acid sequence repeats that characterize several antigens of other protozoan parasites (Enea *et al.,* 1984).

The p67 gene has been expressed as a C-terminal fusion protein with the *Schistosoma japonicum* antigen, Sj-26, using the PGEX expression vector system (Nene *et al.,* 1992). Rat anti-serum raised against the purified Sj26-

fusion protein neutralized sporozoite infectivity *in vitro (Nene et al.,* 1992), but the fusion protein was highly unstable. On testing alternative expression systems, the plasmid PMGI, a derivative of the pAS expression system, was found to be more suitable. PMGI gives rise to the expression of recombinant antigens as C-terminal fusion proteins with NS1(Young *et al.,* 1983), a non-structural protein of influenza virus A. The translational fusion was created by cloning a repaired 2.3 kbp BamHI fragment containing the p67 gene into the *HpaI* site of pMG1. Transcription of the hybrid gene is regulated by a temperature-sensitive mutant of the cI repressor gene of bacteriophage lambda and is driven by the P promoter of *lambda.* Expression of the fusion protein is induced by heat shock and the insoluble protein, termed NSI-p67, contains the first 85 amino acid residues of NSI, two residues encoded by a DNA linker sequence and all 709 residues of p67 (Musoke *et al,* 1992).

IMMUNIZATION AND CHALLENGE OF CATTLE

The antigen for immunization was prepared as described previously (Musoke *et al.,* 1992). Nine Boran *(Bos indicus)* cattle aged between six and eight months, which were serologically negative for antibodies to *T. parva* and had been maintained under strict acaricidal control, were used. The nine animals were immunized by subcutaneous inoculation wiTh1 mg of the antigen preparation, equivalent to 600 μg of NS1-p67, formulated in 3% saponin (Merck). A control group, comprising four animals immunized wiTh1 mg of antigen preparation expressing NS1 and six uninoculated cattle, was included. The nine cattle as well as the four controls received further inoculations of the appropriate antigen at monthly intervals for four months. Ten days after the final boost all the cattle were challenged with one LD_{68} of a *T. parva* (Muguga) sporozoite stabilate 3087.

Pre-challenge sera from the nine NS1-p67 immunized animals contained specific antibody titres in excess of 1:62,000 when analysed by sandwich-ELISA (Katende *et al.,* 1990) using both the native and recombinant antigens and recognized both antigens as well as several *Escherichia coli* proteins on immunoblots (Musoke *et al.,* 1992). The sera had neutralizing antibody titres greater than 1:200 in an *in vitro* sporozoite infectivity neutralization assay (Musoke *et al.,* 1992).

On challenge, six of the nine cattle immunized with NS1-p67, were protected against challenge with only two of them showing evidence of infection. The remaining three animals underwent severe clinical reactions and were euthanized. Total neutralization of the challenge by the four non-reactors was indicated by the absence of schizont antibodies for 60 days after challenge. Furthermore, the day 9 lymph-node biopsies of two of the non-reactors were negative for the presence of *T. parva* DNA when tested using the polymerase chain reaction described by Saiki *et al.,* (1988), suggesting that elimination of the parasites occurred before schizonts were established. In another group of 11 animals, immunized as described above, six were protected against a heterologous stock of *T. parva.* This result confirmed our earlier observations that stocks of *T. parva* sporozoites posses a common conserved neutralization sensitive epitope(s).

The clinical reactions vary, on challenge, from complete protection with no evidence of disease to severe reactions with fatalities. The degree of protection could not be correlated with the titres of antibody as measured by ELISA or the capacity of the sera to effect neutralization of sporozoites. In search of a correlate, we reasoned that animals that exhibit complete protection may recognize different epitopes than those undergoing severe reactions. To address this question, overlapping peptides covering the entire sequence of the p67 molecule were made using pin technology (Chiron Mimotopes). The peptides were 15 amino acids in length and overlapped by seven residues. The peptides were tested in an ELISA system using neutralizing monoclonal antibodies (MAbs) generated against recombinant and native p67 antigens, as well as bovine sera from animals immunized with NS1-p67. The neutralizing MAbs recognized four distinct peptides, with three being recognized by MAbs raised against recombinant p67 and one by TpM12, which was raised against the native molecule.

The bovine immune sera recognized only one of the peptides reactive with the MAbs. The rest of the peptides recognized by bovine sera lay at either end of the molecule and none were recognized strongly in the middle of the molecule.

CONCLUDING REMARKS

The recombinant form of p67 protected 60-70% of the immunized cattle on challenge with an LD_{68} of *T. parva* sporozoites. That protection of the immunized animals was due to the NS 1 -p67 antigen is indicated by the total susceptibility of the animals immunized with a lysate of *E. coli* expressing NS1. In addition, more recent experiments (data not shown) have shown that the purified form of the antigen can induce a much higher level of protection. The immune mechanisms responsible for protection in the immunized animals have not been fully elucidated. The failure of the parasites to establish infection in the four non-reactors in first experiment suggests that the effect is directed either against free sporozoites or recently infected cells. It is likely that the high antibody titres present in the immunized cattle prior to challenge were involved in this effect. However, quantity of the antibody in the immunized cattle did not correlate with protection, suggesting that the efficiency of neutralization may have been influenced strongly by the antibody affinity. It is also possible that T-cell responses directed at the recently infected cell are involved in the protection. There is evidence that p67 is shed by the sporozoite during its entry into the lymphocyte (Webster *et al.*, 1985). It is possible that the shed p67 enters the MHC class II antigen presentation pathway, and renders the recently infected cells susceptible to cytolytic or cytokine-mediated effector function of $CD4^+$ T cells. An influence of cytokines on parasite multiplication *in vitro* has recently been demonstrated (Preston et al., 1992). It is of interest to note that in the closely related *Plasmodium*, both humoral and cell-mediated responses to the circumsporozoite antigen have been shown to confer protection (Tsuji *et al.*, 1990; Orego and Facer, 1993).

For reasons that are not clear, p67-specific T-cell proliferative activity was not consistently detected in immunized animals up to the time of challenge.

However, T-cell responses have been detected in other animals immunized with the native p67, and some T-cell clones from these animals recognize the NSI-p67 molecule. We are currently investigating this anomaly by measuring cytokines in supematants from cultures of cells derived from NS1-p67-immunized animals.

An efficacious vaccine against a parasite with several stages in the mammalian host should ideally stimulate protective responses against all its stages. Partial neutralization of sporozoites may therefore be a desirable feature of a sporozoite-based vaccine against *T. parva* since limited breakthrough of infection will allow generation of protective responses against the schizont stage of the parasite. This was observed to be the case in this study; animals that showed mild clinical reactions as the result of partial neutralization of sporozoites generated potent cytotoxic responses against schizont-infected lymphocytes and were solidly immune to re-challenge with an LD,00.

Since our protocol did not induce protection in all immunized cattle, alternative delivery systems need to be explored. Nevertheless, our results clearly demonstrate that it is possible to induce protective immunity to ECF in cattle with the recombinant p67 sporozoite antigen.

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Development of bovine-specific cytokine reagents at ILRAD

B. Mertens, L. Gaidulis, S. Mwangi, H. Suliman and K.A. Taylor

International Laboratory for Research on Animal Diseases
P.O. Box 30709. Nairobi, Kenya

INTRODUCTION

Infection of livestock with African trypanosomes leads to haematological changes such as anaemia and pancytopenia (Dargie, 1980; Murray and Dexter, 1988) and to immune defects, including suppression of T-cell responsiveness (Sileghem and Flynn, 1992) and an excessive increase in the $CD5^+$ B-cell population in blood and spleen (Naessens and Williams, 1992). It has been shown that certain breeds of cattle have evolved mechanisms to reduce the effects of trypanosome infections (Murray and Dexter, 1988; Paling *et al.*, 1991b). At ILRAD, trypanosome-specific immune responses of trypanotolerant N'Dama cattle *(Bos taurus)* are being compared with those of trypanosusceptible Boran *(Bos indicus)* cattle. The molecular and cellular basis of trypanotolerance are poorly defined, but preliminary data suggest that differences exist between these breeds at the level of T and B-cell responses to trypanosome antigens during infection. Clear differences have been reported between N'Dama and Boran cattle in the quantity, quality and specificity of antibodies produced following trypanosome infection (Paling *et al.*, 1991a, 1991b; Authie *et al.*, 1993a, 1993b). Further characterization of these differences and evaluation of their role in trypanotolerance will require the availability of reagents for the analysis of bovine cytokine responses.

INTERLEUKIN-4

Studies in human and murine systems have shown that IL-4, originally described as B-cell growth and differentiation factor, stimulates the proliferation of T cells and mast cells and is an important regulator for immunoglobulin isotype switch (for review, see Paul, 1991). A cDNA encoding bovine IL-4 (Heussler *et al.*, 1992) was kindly provided by Dr. D. Dobbelaere. The sequences encoding the mature IL-4 molecule were subcloned into the pGEX-3X vector (Smith and Johnson, 1988) and the fusion protein, termed IL-4-glutathione S-transferase (GST), was produced in *Escherichia coli* with typical yields of 3 mg/l bacterial culture. Recombinant IL-4-GST (rIL-4-GST) was tested for biological activity in an assay based on T-cell growth factor activity of IL-4 (TCGF assay). Proliferative responses are determined by measuring incorporation of ^{125}IUDR into concanavalin A-activated bovine peripheral blood lymphocytes cultured for three days in the presence of rIL-4-GST. Maximum proliferation occurred with 50 ug/ml rIL-4-GST while no responses are induced by the GST carrier alone.

The rEL-4-GST was used to immunize mice in order to raise polyclonal and monoclonal antibodies (MAb). Eight hybridomas were selected that recognized rIL-4-GST but not GST in ELISA screening. All eight MAbs are of the IgG l isotype and their ability to block the biological activity of rIL-4-GST is being tested. The availability of recombinant bovine IL-4 and specific antibodies provides useful reagents to investigate T- and B-cell interactions in immune response to infectious diseases in cattle.

TUMOUR NECROSIS FACTOR

In addition to its tumouricidal and immunoregulatory functions, tumour necrosis factoi alpha (TNFα) plays an important role in the control and pathogenesis of infectious diseases. Specific reagents have been developed at IILRAD to study the role of TNFα in the pathogenesis of African trypanosomiasis. Recombinant bovine TNFα (rTNFα), obtained from Ciba-Geigy SA, Switzerland, was used to raise polyclonal and monocional antibodies and these fortn the basis of a bioassay and an immunoassay for TNFα (Sileghem *et al.*, 1992). In addition, the full-length coding region of bovine TNFα has been cloned and expressed in *E. coli* at ILRAD. Biologically active RTNFα and the assays specific for TNFα will be of great value in elucidating the involvement of TNFα in the pathology associated with diseases such as African trypanosomiasis.

HAEMOPOIETIC CYTOKINES/GROWTH FACTORS

Anaemia is a prominent feature of many chronic inflammatory diseases. In livestock, infection with trypanosomes causes a profound decrease in the numbers of circulating erythrocytes and all other blood cell types. The haematological changes during trypanosome-infections have been well characterized and ILRAD scientists have reported differences between trypanotolerant N'Dama and trypanosusceptible Boran cattle in the control of the anaemia (Andrianarivo *et al,* 1993). In contrast, the mechanisms that cause pancytopaenia in trypanosomiasis are poorly understood. One approach to a better understanding of these mechanisms is to study the role of cytokines in the regulation of haemopoiesis. It is possible that the action of some cytokines within the bone marrow of infected animals may adversely affect haemopoiesis. In humans and mice, cytokine-mediated down regulation of bone marrow has been attributed to TNFα, transforming growth factor-beta (TGFβ) and interferon gamma (IFNγ). Conversely, erythropoietin is one of the most important positive regulators of erythropoiesis (for review, see Koury and Bondurant, 1992).

To gain a better understanding of the mechanisms controlling ineffective haemopoiesis in trypanosomiasis, we have started to clone the genes for bovine haemopoietic factors.

Stem Cell Factor

Stem cell factor (SCF), also known as c-kit ligand, mast cell growth factor or steel factor, is an essential regulator for normal haemopoiesis, melanogenesis, gametogenesis and mast cell growth and development. In humans and mice it has been shown that SCF exists in both membrane and soluble forms (Anderson *et al.*, 1990). To clone the bovine cDNA portion encoding soluble SCF, primers were designed based on interspecies homologies. RNA was isolated from bone marrow mononuclear cells of Boran cattle and reverse transcribed into cDNA for use as a template for the PCR reaction. After subcloning and sequencing of various PCR fragments, a cDNA clone was selected with an open reading frame complete to the terminal amino acid residue 174. A 25-residue leader peptide similar to that of human SCF is present and homology with human SCF at amino acid and nucleotide level is 85 and 90%, respectively. Preservation of cysteine residues at positions 4, 43, 89 and 139 most likely results in disulphide bridging identical to that seen in the rodent and primate SCFS. The predicted amino acid sequence of the bovine molecule contains an additional residue at position 130 that is not present in humans or rodents, but is present in the canine analog. Experiments are in progress to express a truncated bovine cDNA encoding soluble SCF, up to alanine residue 165.

Interleukin-3

Interleukin-3 (IL-3) is produced by activated T cells, activated mast cells and granulocytes. This cytokine acts on early progenitors to produce cells of all haemopoietic lineages and modulates the growth and effector functions of mature cells such as macrophages, lymphocytes and mast cells. IL-3 is highly species-specific in its activity. Complementary DNA sequences of human and mouse IL-3 show very low homology: 29% at amino acid level and 45% at the nucleotide level. We have amplified and cloned a 533 bp DNA fragment from cDNA derived from PHA/PMA-stimulated bovine peripheral blood lymphocytes (PBL) using primers based on 5' and 3' untranslated regions of the ovine IL-3 cDNA (McInnes *et al.*, 1993). The bovine cDNA clone contains an open reading frame of 432 bp encoding a protein of 144 residues. Homology with the ovine IL-3 sequence is 85 and 90.5% at amino acid and nucleotide levels, respectively. Experiments are under way to produce recombinant protein, and to identify the biological activity in bovine clonogenic assays.

Erythropoietin

The hormone erythropoietin (EPO) is an important regulator of terminal erythroid differentiation. A blunted EPO response is implicated in many chronic inflammatory diseases (Means and Krantz, 1992) and may also contribute to the chronic anaemia that is characteristic of trypanosomiasis. To address this issue, a cDNA clone containing the complete open reading frame of bovine EPO has been isolated from Boran kidney tissue. Recombinant protein will be produced using an eukaryotic expression system, since glycosylation of human and mouse EPO has been shown to be essential for biological activity.

ANALYSIS OF BOVINE CYTOKINE RNA EXPRESSION

Because techniques for detection of cytokines at the protein level are sometimes not sensitive enough, measurement of cytokine RNA expression can be a useful alternative for the characterization of immune responses *in vitro* or *in vivo.* While assays such as Northern blot and dot blot hybridization are in use at ILRAD, quantities of RNA are often a limiting factor, so we have also installed the semi-quantitative reverse transcription PCR (RT-PCR) technique for a number of bovine cytokines.

Oligonucleotide primers for the amplification of bovine IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, GM-CSF, IFNγ, TGFβ, TNFα, TNFβ and EPO have been generated. These primer sets are being used for amplification of cDNA derived from total RNA. We have tested several published methods for isolating RNA from small numbers of cells and have selected the guanidine thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (1987). In order to compare the level of gene expression between different samples, aliquots are taken during the exponential phase of amplification and products are hybridized on Southern blots with appropriate digoxigenin-labelled internal oligonucleotides. To ensure that equal amounts of cDNA have been used an amplification with beta actin primers is included.

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The use of cytokines in vaccination strategies

M. Campos, R..J. Frenchick* and H.R.A. Hughes*[†]

* SmithKIine Beecham Animal Health
601 West Cornhusker Highway
Lincoln, Nebraska 68521

[†]Veterinary Infectious Disease Organization
University of Saskatchewan
Canada S7H OWO

INTRODUCTION

Recent information on the effects of cytokines (CK) *in vitro and in vivo* and their significance in pathogenesis and immunity has ensured that CK research will be at the center of future prophylactic and therapeutic developments. The practical applications of this accumulated knowledge are now being explored. One area of application that we believe shows great promise is the use of selected CK to improve immune responses to vaccine antigens. The use of CK in this context has already been explored in cattle by testing the adjuvant potential of interleukin-1 (IL-1), interleukin-2 (IL-2), and interferon gamma (IFNγ) in selected antigen systems. However, this application has not reached clinical practice due to a variety of problems intrinsic to the use of these potent biological-response modifiers, including difficulties in formulation and secondary effects that are often associated with their systemic administration.
This paper will address the research developments that have led us to the approaches currently being used to exploit adjuvant properties of CK. In addition, some concepts will be discussed that might assist in the design of more rational application of these mediators to improved vaccination strategies.

PROPHYLACTIC EFFECTS OF SINGLE-DOSE, SINGLE-CK ADMINISTRATIONS

Early assessments of the potential clinical use of CK examined the prophylactic potential of single-CK, single-dose administrations. It was observed that treatment of cattle with IFNγ or IFNα (Barbiuk *et al.*, 1985, 1987, 1991) prior to sequential challenge with BHV-1 *and Pasteurella haemolytica* reduced the severity of clinical signs and mortality. Virus isolation and immunological studies suggested that the beneficial effects obtained after IFN administration were due to immunoregulatory properties rather than the anti-viral activity of these CK (Barbiuk *et al.*, 1985, 1987, 1991; Bielefeldt Ohmann and Martinod, 1990).

Exploitation of the immunoregulatory properties of IFNs to ameliorate clinical manifestations of disease has also been demonstrated in several other experimental models. Treatment of calves with IFN (5 mglanimal) prior to

crowding reduced the incidence of crowding disease (Bielefeldt Ohmann and Martinod, 1990) and administration of IFNγ prior to *Haemophilus somnus* challenge of dexamethasone immunosuppressed calves resulted in reduction of the severity of pneumonias (Chiang *et al.*, 1990). In addition, intramuscular administration of IFNγ to calves prior to infection with *Salmonella typhimurium* reduced the degree of septicaemia (Peel *et al*, 1990) and intramammary infusion of IFNγ 24 hours prior to challenge with *Escherichia coli* reduced the number of infected quarters, prevented death, diminished clinical signs and shortened the duration of infection (Sordillo and Babiuk, 1991). More recently the prophylactic capacity of single-CK, single-dose administrations has also been demonstrated for IL-1, IL-2, and GM-CSF. All three CK were shown to protect mammary glands from the subsequent challenge with *Staphylococcus aureus (Daley et al.*, 1991).

All of these examples clearly demonstrate the prophylactic effect of CK administration in a single-dose regimen under experimental conditions where timing and exposure can be controlled. However, examples of effective prophylaxis under field conditions, where these parameters are uncontrollable, are not as readily available. In one instance where steers were treated with 5 mg IFNα at feedlot entry, the effects of treatment were not as striking as those observed in experimental situations (Babiuk *et al*, 1991). Results such as these suggest that prophylactic approaches using single-dose CK administration regimes require the use of formulation technologies that allow slow- or pulse-release of CK to cater for different windows of disease susceptibility. However, initial attempts to extend the prophylactic effect of CK using a sustained release formulation or repeated injections have not been successful. Administration of IFNα or IFNγ in consecutive injections failed to protect animals from clinical manifestions and mortality in the BHV-1/*Pasteurella haemolytica* model (authors' unpublished observations), and administration of IFNα in a slow-release formulation was able to sustain levels of 2'5'A synthetase but failed to induce a sustained change in flow cytometric profiles associated with IFNα immunoregulatory properties (authors' unpublished observations). Thus, it appears that the immunoregulatory properties of single-dose CK administrations can only be maintained by careful dosage and timing regimens that cannot be achieved with current formulation technologies.

These experiences, along with many others described in the literature, suggest that exploitation of the immunoregulatory properties of CK under field conditions will require careful selection of protocols designed to regulate specific immune functions responsible for protection. Thus CK would be selected for incorporation in a vaccine on the basis of their capacity to enhance a desired immune function.

POTENTIATING VACCINES USING CK

Communication between cells of the immune system is governed by a number of recognition molecules, adhesion molecules, as well as CK. Recognition of antigen and engagement of adhesion molecules can be followed by polarized secretion of CK, influencing their site and mechanism of action. Under natural conditions, CK act as ultra-short range mediators and should be compared more with neurotransmitters than hormones. From the results of an elegant series of experiments, Mitchison (1990) proposed a general scheme for interactions between immune cells. Ibis scheme involves clusters of two or three cells that incorporate either T and B cells (2-cell cluster) or T cells, B cells and interdigitating dendritic cells (3-cell cluster). These cell clusters facilitate the delivery of signals for the activation of effector cells. An excess of CK between an effector cell and a regulatory cell may have functional significance through the activation of bystander reactions, which may be either beneficial or detrimental to the desired immune response. Mitchison proposed that administration of low doses of CK should be beneficial since only those cells already activated by antigen recognition and appropriate second signals would be expanded. Conversely, high doses might activate redundant effector mechanisms that might be ineffectual or detrimental. We have examined the effect of CK dose on the outcome of immunization schemes.

Most CK tested to date have shown at least a modest beneficial effect when administered with recombinant or subunit antigens (Hughes *et al.*, 1991, 1992a) or with killed or modified live vaccines (Reddy *et al.*, 1990; Nunberg *et al.*, 1989). Nonetheless, CK generally exert their adjuvant effect at an optimal dose; high doses can produce adverse effects, while low doses are ineffective. This has been demonstrated with IL-1, IL-2, and IFNγ in different model systems.

The adjuvant activity of IL-1 appears to be both time- and dose-dependent, with the best activity occurring when the CK is administered at approximately the same time as the antigen (Staruch and Wood, 1983). The activity was first attributed to an indirect effect, such as enhancement of IL-2 release from antigen-driven cells, but this has been disputed in a series of experiments where IL-1 enhanced B-cell responses in the presence of antibodies that neutralize IL-2 (Reed *et al.*, 1989). It is more likely that IL-1 acts through its co-stimulatory effects on T cells following antigen recognition. Recombinant bovine IL-1 P has been administered to cattle in conjunction with a modified live BHV-1 vaccine (Reddy *et al.*, 1990). Administration enhanced the cytotoxic activity of peripheral blood cells against BHV-1 infected targets after immunization. This augmented response was apparent at all doses of CK tested (33 ng/kg to 1000 ng/kg). However, augmentation of neutralizing antibodies against the virus occurred only at 100 ng/kg of IL-1, with higher or lower doses having no effect when compared to the control. Likewise, when animals were challenged with live virus, those animals given 100 ng/kg of recombinant bovine IL-1 P shed significantly less virus than those animals given lower or higher doses. These studies indicate that IL-1 can enhance both cellular and humoral mechanisms of immunity and, further, that the dose at which this occurs is critical for optimal effect.

Following early studies of the adjuvant effects of IL-2, it was proposed that it has two distinct mechanisms of action *in vivo*, one being to overcome genetic

low responsiveness and the other being that of a classical adjuvant, i.e. to enhance a response to antigen. The different activities appeared to be related to formulation with IL-2 in oil overcoming genetic low responsiveness and aqueous preparations providing an adjuvant effect (Heath and Playfair, 1992). However, IL-2 has been shown to overcome *Ir* gene control in a manner similar to that in which KLH can overcome genetic non-responsiveness to peptides (Good *et al.*, 1988). These authors concluded that KLH induces a strong but localized secretion of IL-2 that has a direct effect on the proliferation of peptide-specific T cells. They further proposed that delivering erogenous IL-2 acts in the same way by taking the place of the KLH-induced IL-2 secretion. Thus, the ability of IL-2 to overcome genetic non-responsiveness was not due to formulation; rather, it was due to a localized over-production of CK. Few studies have been carried out in which these diverse activities of IL-2 have been examined simultaneously. In all of those cases where genetic non-responsiveness was examined, the adjuvant effects of the CK in normal (responding) animals was not considered. Further, in all but a few studies, the dose of CK that was administered was extremely high (up to 1 mg/kg). These experiments have been adequately summarized elsewhere (Hughes and Babiuk, 1992). Very few studies have examined a dose response effect of IL-2 in conjunction with vaccines. However, in those instances where this has been done, it is clear that high doses of IL-2 can induce suppression of the specific response, whereas lower doses induce an adjuvant effect. An appropriate dose of IL-2 can induce up to a 25-fold increase in the immune response to killed rabies vaccine (Nunberg *et al.*, 1989). When extremely low doses of IL-2 (0.5 μg/Kg) were given in a multiple dose regimen to cattle following vaccination with authentic glycoprotein D (gD) isolated from BHV-1, there was a 25-fold increase in the immune response to the antigen (Hughes et *al.*, 1991). At this dose of IL-2, no other detectable biological effect can be determined (Campos *et al.*, 1992). This may indicate that the desired effect, enhanced specific activation in the absence of a non-specific bystander response, is being achieved.

A similar trend has been observed with the use of IFNγ in vaccination protocols. In a series of studies, Heath and Playfair have demonstrated that there is a strict optimum dose for the adjuvant effect of IFNγ. When IFNγ was administered in excess, there was either no effect or suppression of the response (Playfair and DeSouza, 1987).

As mentioned earlier, initial work in the use of CK as adjuvants was based on the principle that the CK should be present simultaneously with antigen to ensure that antigen reactive cells were driven to respond. Repeated administration of IL-2 improved the efficacy of a HSV-2 gD vaccine when IL-2 was given throughout the course of the 17-day vaccination schedule (Nunberg *et al.*, 1989). Realization of the impracticality of this type of schedule led several investigators to prolong the presence of the IL-2 through encapsulation, PEG-modification or incorporation in liposomes (Heath and Playfair, 1992). The last two approaches were shown to be effective as adjuvants in vaccine formulations. The PEG IL-2 formulation was effective in providing protection to mice from rabies at a lower dose (0.05 versus 0.1 μg) and with fewer injections (1 dose/day versus 2 doses/day) but the CK still needed to be administered for five days (Nunberg *et al.*, 1989). A liposomebased IL-2

formulation also provided protection in mice against challenge with influenza A virus, suggesting that both cellular and humoral responses can be effectively stimulated using this approach (Heath and Playfair, 1992). In addition, improvement of antigen-specific humoral responses has been observed after administration of IL-2 in an emulsion formulation that is believed to extend the lifetime of the entrapped IL-2 (Heath and Playfair, 1992). It is possible that liposomes and emulsions activate phagocytic cells and cause the local release of other CK capable of acting in synergy or counteracting the administered IL-2. Therefore, the results of these studies may not be solely due to the effect of administered IL-2. The examples provided above demonstrate the benefits of prolonging the CK effects through formulation. However, extending the circulating lifetime of the CK does not limit its activity to a specific location such as a regional lymph node. The CK may still stimulate inappropriate immune responses in potentiating the response to the vaccine.

When doses and localization of CK are uncontrolled, there is a danger that bystander reactions could lead to autoimmune disease. This has been observed in an experimental model as well as in patients who have been given large doses of CK (IL-2 or IFNα) for treatment of renal cell carcinoma and melanoma (Scaizo *et al.*, 1990). These data have indicated that IL-2 may have the ability to overcome tolerance to auto-antigens, with resulting autoimmune disease. Indeed, IL-2 has been termed a 'pro-autoimmune' lymphokine (Kroemer and Martinez, 1992). These observations have led investigators to associate the CK with antigen through chemical linkage or by co-expression in live vectors to guarantee delivery of the CK to the same local environment as the vaccine antigen. This approach not only ensures that CK and antigen reach the correct destination at the same time, but it also allows the dose of CK to be optimized by this method so that activation of bystander reactions does not occur. Results from virus challenge following immunization with CK/antigen-double vaccinia recombinants demonstrated that there was no reduction in immunogenicity of either the vaccinia antigens or the recombinant antigen in the case of IL-2/HSV/vaccinia and IL-2/influenza/vaccinia constructs. However, co-expression of the CK did not provide any improvement in protection when compared to the expression of antigen alone (Heath and Playfair, 1992). Therefore, direct linkage of antigen with a CK may hold the greatest promise for the development of vaccines that use the CK as immune potentiators. Association of CK with antigens has been accomplished through chemical coupling, genetic fusion, or the formation of ternary complexes such as CK-biotin-avidin. The CK used in these formulations have been an IL-1 β nonapeptide, IL-2, IFNγ, and GM-CSE

The initial attempts to associate CK with non-viable antigens used an IFNγ-biotinavidin complex as an immunogen without additional adjuvant. This complex induced increased anti-avidin antibody titres and significantly improved the DTH response to avidin as measured by ear swelling. These data suggested that the expected Thl response was induced. However when the anti-avidin antibody responses of Gl and G2a subclasses were examined, there was no statistical difference between the group immunized with the complex and the control group.

Fusions or chimaeric molecules incorporating antigen and CK components have provided interesting results. It has been shown *in vitro* that the bioactivity

of IL-1, IL-2, and GM-CSF in such molecules is retained, providing encouragement for their potential in *vivo* effects. IL-2 has been genetically fused to the HSV-1 gD and the *lkt*A gene product *from Pasteurella haemolytica* and used to immunize animals (Hughes *et al.*, 1992a; Hazama *et al.*, 1993a). In the latter study cattle were immunized with the chimera in combination with an emulsion adjuvant, using a multiple step immunization schedule. No improvement of either the humoral or cellular immune response was observed when compared to that induced by the *Ikt*A antigen alone. However, the adjuvant used in these studies was a strong inducer of immune responses to *lkt*A, so that detection of an IL-2-related improvement was difficult.

In the HSV-1 gD study, the fusion protein was administered to mice either parenterally or intranasally, without other adjuvants (Hazama *et al.*, 1993a, 1993b). Following subcutaneous immunization with the gD-IL-2, specific antibody titres, DTH responses and protection were equal to that induced by the gD formulated in complete Freud's adjuvant. The adjuvant effect of the fused IL-2 was not extended to an additional antigen incorporated in the inoculum. The use of the same IL-2-gD protein administered intranasally was also examined. The chimera was a better primary immunogen than gD or a mixture of IL-2 and gD and induced significant protection to intranasal challenge with HSV-2.

An alternative approach for the generation of immunogenic chimaeric molecules used peptide synthesis to link a nonapeptide derived from IL-1β and hepatitis B S1 antigen (Kanury and Nayak, 1990). Immunization with this synthetic chimaera adsorbed on aluminum hydroxide improved the number of mice with anti-peptide antibodies following primary immunization and increased the final anti-peptide titre when compared to those in mice immunized with a mixture of antigen and CK. The enhanced antibody response was seen in mice of four different haplotypes. A separate study examined the effect of genetic insertion of the same IL-1β peptide into the immunizing proteins (Beckers *et al.*, 1993). The immunogenicity of these recombinant proteins was evaluated in the absence of other adjuvants. Specific responses were significantly greater to the chimaera than to control proteins over the period form day 4-38 after primary immunization.

It is clear that CK are effective adjuvants and that the delivery of antigen and CK to the same cell or micro location can further improve the immune response while limiting untoward stimulation of other immune cells. The use of other adjuvant compounds in some of the studies has made analysis of the exact role of the CK in these vaccine formulations difficult. However, in most cases it appears that the addition of the CK to antigens improves both humoral and cellular responses. The results do not conform to the current paradigm for the role of CK in directing immune responses to exclusive pathways. It was originally thought that administration of a Th1 CK such as IL-2 or INFY would result in a Th1 -type responses against the immunizing antigen. Similarly, it was assumed that incorporation of Th2 CK such as IL-4 or EL-5 in a vaccine would enhance certain type of antibody responses. While the Thl-Th2 paradigm is clearly significant in the pathogenesis of certain diseases and may be relevant to the use of CK therapy in a limited number of infectious diseases (Yamamura *et al.*, 1991), it appears that it does not apply to the use of CK as immunological adjuvants with vaccines. We have observed that when IL-2 is administered with

a subunit antigen, it does not appear to influence the isotype specificity of the humoral response or to shift the response towards cellular or humoral elements (Hughes *et al.,* 1991, 1992b). Indeed, all responses were equally enhanced. Similarly, administration of IL-1 enhanced both humoral and cellular immunity against a modified live B HV-1 vaccine (Reddy *et al,* 1990). Furthermore, when a HSV gD-IL-2 chimeric molecule was administered intranasally, there was an enhanced lgA production in the lungs of mice. In this case, a Thl CK was apparently enhancing a Th2 response (Hazama *et al.,* 1993b). Other workers immunized mice with liposomes containing antigen and IL-2 or IL-4 and, contrary to the current paradigm for mucosal T-cell help, observed that only the IIL-2-containing liposomes enhanced antigen-specific lgA responses (Abraham and Shah, 1992). Ibus the use of CK as immunological adjuvants depends on many factors including the nature of the antigen, the dose of CK, the route of administration, and the formulation used. All of these factors may be as important to the outcome of the immunization as the CK itself.

With the exception of the viral vectors, the formulations described above do not target antigen to either pathway for association with MHC molecules. The use of formulations based on acid-sensitive liposomes (Reddy *et al.,* 1992), iscoms (Taskahashi *et al.,* 1991), or certain adjuvants (Wu *et al.,* 1992) that allow antigen association with MHC class I molecules in conjunction with CK has not been reported to date. Use of CK in association with these delivery systems might alter the broad immune enhancement so far observed with CK. This approach may allow more predictable specific induction of a desired immune response. Thus it may be possible in the future to use a CK associated with neutral liposomes containing an antigen to induce an IgGI response, while a different CK associated with acid-sensitive liposomes containing an antigen might induce a strong DTH and CTL response, with neither formulation enhancing the alternative response.

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Summary of discussion

Chairperson: *D.J.L. Williams*
Rapporteurs: *S. Morzaria and K. Taylor*

This session dealt with how appropriate immune responses can be enhanced through the design system. Four papers were presented that described how different responses of the immune system to the same challenge can influence the outcome of infection or immunization with regard to pathogenesis or protection. The issue of influencing the outcome of an immune response through the use of defined delivery systems and cytokine reagents was also addressed.

The plasticity of the immune response received some discussion. To identify the mechanisms involved in protective immune responses, it was clear that detailed analysis of the roles of different components of the immune system is necessary. The use of *in vivo* experiments based on gene knockouts, cytokine treatment, administration of neutralizing antibody and depletion of immune cell subsets has yielded valuable information on the role of various immune cell types in protection and pathogenesis in a number of experimental systems. However, it should be noted that compensatory mechanisms can be invoked through the experimental removal of the major arm of the normal responses to a given immunological challenge, and so results of these experiments must be interpreted with care. It was clear from the experiences at ILRAD with the use of *in vivo* depletion techniques in cattle that the application of these methodologies in this species is still subject to a number of technical problems.

The group focused for a period on the significance of the fine specificity of antibody responses to the outcome of immunizations and infections. In particular, a distinction was made between the epitope specificities of NIAB and those of polyclonal antisera raised by immunization. The distinction was highlighted by an observation made in the murine malaria system where a MAb that recognizes a 3 amino acid epitope of the circumsporozoite protein neutralizes sporozoite infectivity, whereas immunization with the full length protein fails to protect. This may reflect steric hindrance of this neutralizing epitope by other antibodies, but does highlight the importance of immunizing against the protective epitope.

Emphasis was placed on the importance of antigen delivery to the outcome of immunization. Events occurring at the point of delivery determine the nature of the resulting immune response. Great care must therefore be taken in the selection of the site of inoculation and the delivery system or adjuvant preparation to be used.

It was pointed out that experiences in the respiratory syncitial virus (RSV) system indicate that the manner in which individuals are immunized can also profoundly affect the outcome of a challenge infection, with certain immunization regimes being associated with enhanced lung pathology. The latter appears to relate to a Th-2 type response.

Considerable discussion focused on the results of immunizations performed at ILRAD with the p67 surface antigen of *T. parva* sporozoites. Levels of protection already achieved in the face of needle challenge were considered highly promising, and the discussion turned to possible ways in which this could be improved. It was agreed that future research should aim towards identifying immunological parameters that correlate with protection and generating a challenge dose that will mimic field challenge.

A final point was made that ILRAD should continue to develop new reagents necessary for immunological research in cattle rather than restricting its efforts to areas determined by available reagents.

Concluding discussion

The concluding discussion was organized around summary statements prepared by the chairperson of each session of the meeting, which identified the key themes and questions that arose from the session.

SESSIONS I AND II

Initial discussion of this part of the programme focused on whether it was actually necessary to complement a p67-based vaccine with antigens that would provoke cytotoxic T lymphocyte responses. It was pointed out that the combined complexities of *Theileria parva* and the bovine MHC might pose severe problems to the design of a vaccine that would stimulate different arms of the immune response against distinct stages of the parasite, and that it might therefore be more prudent in the short term to explore further the potential of the p67 antigen.

The discussion then turned to the state of knowledge of the bovine MHC and the value of peptide stripping as opposed to screening of cDNA libraries for the identification of antigens that constitute targets for CTL. Examples of the identification of such antigens in virus systems were considered too simple and possibly invalid in the context of *T. parva,* and it was suggested that tumours were perhaps more analogous to this system. The predominant concern was that technologies for the isolation of CTL peptides were very new and had yielded only limited numbers of peptides that might have promise as vaccine candidates. It was nonetheless recognized that CTL had been demonstrated to provide protection against *T. parva,* and that the technologies for peptide isolation and cDNA screening were now established at ILRAD with collaborations in place with the leading laboratories in the area. All of these elements should maximize the likelihood of identifying candidate antigens. It was also pointed out that the number of schizont transcripts was estimated at no more than 4000 and that there had been important developments in the study of bovine class I and II MHC that would complement the search for antigens. A general consensus was reached that ILRAD should continue its search for CTL epitopes using peptide stripping in conjunction with other approaches.

A point about the value of mouse models and the use of knock-out mice in identifying protective responses led to a lively discussion on their possible use in the systems of relevance to URAD. 7lis was largely driven by the report of difficulties encountered in eliminating all cells of a particular population during depletion experiments in cattle. here was no question of the value of mouse models in establishing mechanisms of immunity; the example of helper T-cell responses in *Leishmania* was a good one in that a set of rules had been established that could be extrapolated to other species. However, it was not considered realistic for ILRAD to become involved in in-depth studies in mice in the context of trypanosomiasis research. Large numbers of quality cattle of defined breed and genetic background and the capacity to work with

monozygous and chimaeric twins were perceived as a major resource of the Institute that confer considerable comparative advantage.

SESSIONS III, IV AND V

Discussion on the search for a vaccine against trypanosomiasis focused on both anti-parasite and anti-disease approaches. Differences between Boran and N'Dama cattle in their responses to infection were listed (Table 1) as an aid to discussion and major areas of debate were the role of parasite load in the pathogenesis of the disease and the significance of the failure of the more susceptible Boran cattle to effect isotype switching during their response.

It was observed that the endocrinological failure described in trypanosomiasis closely resembles that seen in malaria, where *Plasmodium* antigens disturb the insulin signalling pathway. The possibility was raised that trypanosome antigens might induce a similar perturbation, perhaps resulting in pituitary dysfunction. A role for cytokines or hormonal reactions in metabolic and physiological disturbances can be investigated, even in such a complicated disease as trypanosomiasis. It was suggested that a single mechanism might control parasite load, so that an anti-disease approach could complement an anti-parasite approach.

It was stressed that the more we can learn about cytokines, the more we will learn about immunology and disease. Massive disturbances of the immune system occur in both trypanosomiasis and ECF, and few, if any, investigations are being conducted elsewhere on the involvement of cytokines under such circumstances. The capacity at ILRAD to explore bovine cytokine function was perceived as impressive and of great value in dissecting the relative roles of TNF, IFNγ and IL-1 in damage associated with acute phase responses. This information is important for determining whether the pathology of these diseases arises from the parasite or the host response. It was pointed out that the striking differences in immunoglobulin responses between Boran and N'Dama cattle may be related to immune memory as well as isotype switching; this will be reflected in the cytokines produced by T cells and other regulatory cells in response to challenge, and will become apparent as the investigations progress. It was proposed that the trypanosome cysteine protease, a molecule that might induce pathogenic effects, could be used as a tool to investigate the disturbances in the immune response. The possibility that degradation products of antigens such as this might be responsible for the pathology should not be ruled out.

Table 1. Differences between N'Dama and Boran cattle in their responses to infection with *Trypanosoma congolense*.

Clinical parameter	N'Dama	Boran
Parasitaemia	lower	higher
Anaemia (PCV)	less severe	
Neutropaenia	less severe	

Antibody responses	I_gm	I_gGI	I_gG2	I_gm	I_gGI	I_gG2
Congopain	+	++	-	+	-	-
BiP (hsp70)	+	++	-	++	+	-
VSG (cryptic)	+	++	-	+	+	-
SEE-VSG	+	+	+	+	+	+
β-Galactosidase	-	-	-	++	-	-

Macrophage Function		
IL-1/1L-6 Activity	+ earlier peak	+
Trypanosome phagocytosis	+ earlier peak	+
Erythrophagocytosis		+
T-cell proliferation	++	+
$CD5^+$ B-cells	+	+
Serodeme-specific acquired immunity	++	+

While the functional role of γδ T cells is still poorly understood, it was acknowledged that the results of studies at ILRAD complements those emerging from other laboratories. Considerable numbers of these cells occur in the spleens and peripheral blood of young cattle and since young animals are the principal target for improved vaccines, elucidation of the role of this population in bovine immunity is an important goal. A thorough investigation of their role in trypanosomiasis and ECF was considered justified. It was pointed out that cytotoxicity was not the only mechanism of controlling intracellular parasites. There are many examples of protection being mediated by inhibition of growth, evidence for this has been presented for both trypanosomiasis and *Theileria* parasites. Hence, γδ T cells need not necessarily act through cytotoxicity but could exert their function by inhibition of growth.

The issue of reversed age resistance in which young cattle can tolerate or control parasite infection and develop immunity in the absence of disease was raised, and it was speculated that γδ T cells may be involved in this phenomenon. It was also asked whether natural killer (NK) cells might be involved. These cells are present in cattle and have a $CD2^+$ phenotype but do not express CD3, CD4 or CD8. They are present in very low numbers in blood and are readily activated to kill by cytokines. A minor role has been attributed to them in killing *T. parva*-infected cells.

A brief discussion on the plasticity of the immune response ensued despite some confusion as to its definition. The meeting concluded that the research programmes at ILRAD should focus on the major effector mechanisms in the target animal population and the identification of the parasite antigens that provoke them.

It was emphasized that development of the p67 antigen of *T. parva* as a vaccine will proceed in conjunction with a commercial partner. The mandate of ILRAD is to identify protective antigens and confirm their vaccine potential by presenting them effectively to the target the animal. Vaccine production and quality control will be the responsibility of the commercial partner.

Finally, it was indicated that studies in the field of cell biology would contribute to the search for antigens through enhanced knowledge of the parasite and its interactions with the host. This information could highlight those molecules that are likely to constitute targets for CTL and so reduce the number of antigens to be screened for vaccine potential.

Conclusions and recommendations

- The p67 antigen of *Theileria parva* is an extremely promising vaccine candidate antigen and its potential should be explored vigorously. Although the first promising candidate antigen may not prove to be a successful vaccine, these efforts will considerably enhance the capacity of the laboratory in vaccine development so that further antigens will be exploited rapidly.
- The search for schizont antigens should be pursued through peptide stripping, cDNA screening and other approaches. A successful vaccine for *T. parva* may require stimulation of different compartments of the immune response by antigens of different stages of the parasite.
- Existing studies of bovine MHC should be continued and expanded to provide enhanced support for immunological studies and immunization trials, and for the identification of protective antigens.
- Investigations of the role of cytokines in immunity, parasite control and pathology should be expanded in studies of both *T. parva* and trypanosome infections.
- The search for a trypanosome vaccine should focus on both anti-parasite and anti-disease strategies.
- Studies of the cell biology of both *T. parva* and trypanosomes should be expanded to provide fundamental information towards a better understanding of the parasites and host-parasite interactions and greater opportunities for identifying potentially protective antigens.

APPENDIX:
LIST OF PARTICIPANTS

List of participants

Authié, E.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Campos, Manuel
Smithkline Beecham Animal Health
601 West Cornhuster Hwy
Lincoln, NE 68521-3596, USA

Cox, F.E.G.
Division of Life Sciences
Kings College London
Campden Hill Rd
London W8 7AH
UK

Daubenberger, C.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Dolan, T.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Elliot, T.
Institute of Molecular Medicine
John Radcliffe Hospital
University of Oxford
Headington
Oxford OX3 9DU
UK

Gaidulis, L.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Gardiner, P.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Gray, A.R.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Kemp, S.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Logan-Henfrey, L.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya
Louis, J.A.
World Health Organization
Immunology Research & Training Centre
Luasanne Section
Institut de Biochimie
Univertsite de Laisanne
Ch. des Boveresses 155
CH 1066 Epilanges
Switzerland

Lutje, V
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

McHugh, N.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

McKeever, D.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Mertens, B.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Minoprio, P.
Institut Pasteur
28 Rue du Roux
75724 Paris Cedex 15
France

Morrison, W.I.
Institute for Animal Health
Compton
Nr. Newbury
Berkshire
UK

ole MoiYoi, O.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Morzaria, S.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Musoke, A.J.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Naessens, J.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Nene, V
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Parham, P.
Department of Cell Biology
Stanford University
Fairchild Building D157
Stanford
CA 94305
USA

Playfair, J.H.L.
Department of Immunology
University of College and
Middlesex School of Medicine
Arthur Stanley House
40–50 Tottenham Street
London W 1 P 9PG
UK

Scheerlinck, J.-R
International Laboratory for Research
on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Sileghem, M.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Skilton, R.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Stauss, H.J.
Tumour Immunology Unit
UCL Medical School
91 Riding House Street
London W1P 8BT
UK

Taracha, E.L.N.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Taylor, K.A.
International Laboratory for Research on Animal Diseases

P.O. Box 30709
Nairobi
Kenya

Teale, A.J.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Weiss, W.
Kenya Medical Research Institute
Kisumu
Kenya

Wells, C.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Williams, D.J.L.
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya