T-CELL RESPONSES DURING *TRYPANOSOMA BRUCEI* INFECTIONS

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This thesis is presented in submission for the degree of Doctor of Philosophy in the Faculty of Science.

September, 1997

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SUMMARY

African trypanosomiasis is caused by *Trypanosoma brucei* and is a disease of considerable importance causing infection in both humans and livestock. There is a degree of protection from the antibody response produced by the host but it is ineffective and infections are chronic and debilitating. This chronicity is due to antigenic variation by the trypanosomes and to immunosuppression of the host.

Antigenic variation is a classic, and well studied, B-cell evasion mechanism in a number of infections and can be observed at a highly sophisticated level during African trypanosome infections. As yet, it has been little studied as a possible T-cell evasion mechanism and this is the subject of my thesis.

Initially, a reliable *in vitro* assay system was devised in order to examine the proliferative T-cell responses of mononuclear splenocyte populations as T-cell proliferation had not been detected during trypanosome infections in previous studies. Responses against trypanosome lysates, paraformaldehyde-fixed and live trypanosomes were examined with paraformaldehyde-fixed trypanosomes being the preferred choice of antigenic stimuli. The optimal conditions for this assay were also determined as far as mitogen concentration, concentration of trypanosome antigen and incubation time were concerned.

Using this reliable proliferation assay system I examined a number of trypanosome infection and immunisation regimes. Most of these analyses were conducted using splenocytes taken from mice at first peak of parasitaemia employing parasite lines in each of which 99% of the trypanosomes present express the same variant antigen type (VAT) during the first parasitaemic wave. The mononuclear splenocytes from infected mice produced a high level of proliferation in response to mitogen stimulation but also produced trypanosome antigen-driven proliferation. This antigen-driven proliferative response was mainly against the homologous VATs but there was also a degree of heterologous antigen-driven proliferation in response to some VATs but not against others. Concomitant with these proliferative responses, increased numbers of T-cells per spleen were detected, comprising both CD4 and CD8 populations. Increased numbers of

activated (CD25 expressing) CD4 and CD8 cells were also observed. There was mitogen-driven and antigen-driven Interferon-γ (IFNγ) production, no detectable Interleukin-4 (IL-4), IL-5 or IL-6 and very low levels of IL-2 at the peak of parasitaemia. Mitogen-driven IL-2 production was observed to be maximal 24 hours post-infection. Immunisation with paraformaldehyde-fixed trypanosomes resulted in proliferative responses similar to those seen by the infected mice but there was detectable IFNγ production only in response to mitogen, not after antigenic stimulation. Further experiments were carried out using paraformaldehyde-fixed ILTat 1.3 or GUTat 7.1 trypanosomes for immunisation followed by homologous or heterologous infection. There was a degree of protection towards challenge with homologous VAT in both cases but also some cross-protection against heterologous VAT. Strong proliferative and IFNγ responses mirrored these results following stimulation with mitogen, homologous and heterologous VATs.

Analyses of the amino acid sequences for several different variant surface glycoproteins (VSGs) (the proteins that determine VATs) were carried out using a computer software package, "TSites". This highlighted potential T-cell epitopes on the VSG sequences using four algorithms separately. Using a set of self-determined guidelines, predicted T-cell epitopes for each of the sequences were mapped onto the MITat 1.2 tertiary VSG structure.

Four peptides, two from the alpha helices and two from loop regions of the ILTat 1.3 and ILTat 1.61 sequences, were synthesised using the data supplied from sequence analyses and epitope mapping. These peptides, conjugated to carrier proteins, were used for a number of immunisation and infection regimes. Proliferative responses and cytokine production were examined following immunisation and then infection with ILTat 1.3 or ILTat 1.61 trypanosomes.

Immunosuppression is considered to be a major contributing factor in continuation of an infection by dampening down any effective immune response during African trypanosomiasis. Immunosuppression is noticeable after the first peak of parasitaemia and involves a number of components with 'suppressor' macrophages appearing to play a key role. I therefore investigated the role of nitric oxide (NO), a major product of

activated macrophages, in the regulation of T-cell responses during chronic *Trypanosoma brucei* infection. Using transgenic mice deficient in inducible nitric oxide synthase (iNOS), the proliferative and cytokine responses were examined. Daily parasitaemias were determined, nitrate levels calculated and antibody isotyping examined using infected and uninfected mice homozygous and heterozygous for the iNOS loci. This work highlighted the key role of NO in the regulation of T-cell responses during trypanosome infections: in the absence of iNOS activity there was an upregulation in proliferation, IFNγ production and IL-2 receptor expression causing improved clearance of trypanosomes from the systemic circulation.

In conclusion, this body of work has successfully designed an *in vitro* assay system to examine T-cell proliferation to trypanosome VATs and, using this assay, I have successfully detected trypanosome antigen-driven T-cell proliferation during acute infections. This proliferation was observed using mononuclear splenocyte populations from infected and immunised mice and was found to be homologous antigen-driven and of T-helper 1 type. There was however heterologous antigen-driven T-cell proliferation against some trypanosome VATs but a lack of proliferation against others. A pivotal role for NO in causing immunosuppression during chronic *T.brucei* infections was determined and this immunosuppression influenced the cytokine production and T-cell proliferative responses but did not appear to affect the antibody production. These data suggest that VAT-specific T-cell responses are an important component of the immune response to *T.brucei*.

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ANTat	Antwerp *Trypanozoon* antigen type
APC	Antigen presenting cell
CBSS	Carter's balanced salt solution
CO_2	Carbon dioxide
Con A	Concanavalin A
DAPI	4, 6-diamino-2-phenylindole
DNA	Deoxyribonucleic acid
EATRO	East African Trypanosomiasis Research Organisation
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol-bis-(β-amino-ether) N', N'-tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
g	Acceleration due to gravity
GUP	Glasgow University Protozoology
GUTat	Glasgow University *Trypanozoon* antigen type
HIV	Human immunodeficiency virus
IFNγ	Interferon-γ
IL	Interleukin
ILTat	International Laboratory for Research on Animal Diseases, *Trypanozoon* antigen type
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
KLH	Keyhole limpet haemocyanin
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
MAb	Monoclonal antibody

μCi	Microcurie
MHC	Major histocompatibility complex
MITat	Molteno Institute *Trypanozoon* antigen type
NK	Natural killer
NO	Nitric oxide
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PHA	Phytohaemaggluttanin
PSG	Phosphate buffered saline with 1% glucose
PWM	Pokeweed mitogen
RBCs	Red blood cells
RNI	Reactive nitrogen intermediates
SAPU	Scottish Antibody Production Unit
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNAP	S-nitroso-acetyl-penicillamine
TGFβ	Transforming growth factor-β
TNFα	Tumour necrosis factor-α
VAT	Variable antigen type
VSG	Variant surface glycoprotein
WHO	World Health Organisation

DECLARATION

This thesis is submitted in accordance with the regulations for the degree of Doctor of Philosophy in the University of Glasgow. No part of it has been previously submitted by the author for a degree at any university. The results presented in this thesis are my own except where stated otherwise.

Some of the work in Chapters 4 and 5 has been published as meeting abstracts:

A.E. Millar & C.M.R. Turner (1995) Antigenic variation as a T-cell evasion mechanism in *Trypanosoma brucei* infections. *Immunology,* **86,** 138.

A.E. Millar & C.M.R. Turner (1995) Antigenic variation as a T-cell evsion mechanism in *Trypanosoma brucei* infections? *Transactions of the Royal Society of Tropical Medicine and Hygiene,* **89,** 591.

ACKNOWLEDGEMENTS

First of all I'd like to thank Dr. Mike Turner for his helpful advice and encouragement throughout this project.

I would also like to take this opportunity to thank Dr. Jeremy Brock for his encouragement and support over the years; Drs. Allan Mowat, Catherine Lawrence and Paul Garside for their helpful discussions; and Dr. Charlie McSharry for teaching me the finer aspects of flow cytometry. I acknowledge the financial support of the Medical Research Council.

I'd like to thank Sam for always being there for me, no matter what, and of course for all the beers; and a big thank you to Mark for all his help and support in the printing of this thesis.

Finally, I'd like to dedicate this thesis to my Mum, Gran and Papa for believing in me, for their continued support and for always saying the right thing at the right time.

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"What lies before us and what lies behind us are
small things compared to what lies within us"
- Ralph Waldo Emerson

CHAPTER 1

GENERAL INTRODUCTION

1.1. *TRYPANOSOMA* PARASITES

The genus *Trypanosoma* covers a large range of parasitic protozoa found throughout the world in a variety of different hosts. A few of these species are pathogenic whereas the majority do not produce disease. The pathogenic species are responsible for a range of diseases known collectively as trypanosomiases and it is the tsetse-transmitted trypanosomes of sub-Saharan Africa and the reduviid-transmitted trypanosomes of South America that create most concern for man. In Central and South America, at least 20 million people are infected by *Trypanosoma cruzi*, the causative agent of Chagas' disease, per year. The annual human infection rate by trypanosomes in Africa is far lower with an estimated 25 thousand new cases of sleeping sickness reported each year (WHO,1986) but this almost certainly a gross underestimate (Pepin,1997). Human sleeping sickness is caused by either *Trypanosoma brucei gambiense* or *Trypanosoma brucei rhodesiense* and both are fatal if untreated. In Africa the problems posed by trypanosomiasis are difficult to resolve (reviewed by Kuzoe,1993). Trypanosomiasis can have a direct effect on humans, whereby they cause disease in humans, and an indirect effect, where the parasites infect livestock. The indirect effect is caused by trypanosome infections of domestic livestock by *Trypanosoma congolense*, *Trypanosoma vivax* or *Trypanosoma brucei brucei*. These diseases of livestock are collectively termed "nagana". The infected animals can cause economic problems for farmers due to the loss of production, infertility, increased abortions and wasting. Areas of South America and Southern Asia have trypanosome-infected animals, arising mainly from *T.evansi*, although this is not due to the tsetse fly but rather to non-cyclical, contaminative transmission by tabanid flies or by carnivores eating infected carcasses.

This project involves tsetse-transmitted trypanosomes from the *T.brucei* species of sub-Saharan Africa. *T.b.brucei*, *T.b.gambiense* and *T.b.rhodesiense* are the three subspecies of this group. *T.b.brucei* is not human infective, due to a substance present in human serum which is lytic to these parasites, whereas *T.b.gambiense* causes the more chronic infection in West Africa, and *T.b.rhodesiense*, causes the more acute infection in Eastern Africa (Bentivoglio *et al*,1994). The three subspecies are however morphologically indistinguishable from each other. Nevertheless, *T.b.gambiense* could be considered a separate species due to its differences in ribosomal RNA genes and isoenzymes (Gibson *et al*,1980; Tait *et al*,1984) compared with the close phylogenetic relationships of *T.b.brucei* and *T.b.rhodesiense* with each other.

1.2. THE LIFE CYCLE OF *T.BRUCEI*

T.brucei trypanosomes have a common life cycle which involves a mammalian host and transmission via an insect vector into another host. The life cycle alternates between dividing forms, which cause establishment of infection in a tsetse or mammalian host, and non-dividing stages, which are associated with the transmission of the infection between mammals and vectors (Vickerman,1985). During the life cycle, the trypanosomes alter their morphology, energy metabolism and biochemistry as a pre-adaptation mechanism to allow survival in their current environment (Opperdoes,1985).

The transmission of trypanosomes is by the tsetse fly (*Glossina* species) insect vector with the tsetse fly inhabiting 11 million kilometres^2. The palpalis group transmits *T.b.gambiense* whereas the morsitans group is responsible for the transmission of *T.b.rhodesiense*. Non-dividing mature metacyclic trypanosomes enter the mammalian host in the tsetse fly saliva when the infected fly has a blood meal. The metacyclics differentiate rapidly to slender forms that divide by binary fission and establish the infection in a new host. These long slender forms establish systemic infections in the mammal and the infection continues through evasion of the host immune response by the process of antigenic variation

(Vickerman,1985). The basis for antigenic variation is a surface coat of variant surface glycoprotein (VSG). Slender forms give rise to short stumpy forms that differ from them in that they are non-dividing and have a finite life span unless they are taken into a tsetse midgut with a blood meal where they can transform to dividing procyclic trypanosomes. Slender forms are not able to infect tsetse flies. On entering the fly, the trypanosomes replace their VSG surface coat with a surface coat of procyclin. The procyclic trypanosomes become non-dividing proventricular forms which migrate from midgut to salivary glands where they change to dividing epimastigotes, attached to the endothelium in the salivary gland. When the epimastigotes transform into non-dividing metacyclics, the procyclin becomes stripped and new VSG is synthesised on the metacyclic forms in preparation for life in the mammalian host (Vickerman,1985; Cross,1990).

Changes in the trypanosomes' environment during the life cycle results in changes in their energy metabolism. The slender forms obtain energy by glycolysis, absorbing glucose from the host bloodstream to generate ATP and releasing pyruvate as a waste product, and not via the Kreb's Cycle as they do not have a functioning electron transport chain (Vickerman,1985). These bloodstream forms carry out their glycolysis in glycosomes which house the glycolytic enzymes of the trypanosome. However this is not the case for the stumpy forms which have a partially activated electron transport chain. When they enter the tsetse fly midgut they encounter an environment that is rich in proline but lacks glucose, therefore the procyclic trypanosomes utilise this energy source by way of the electron transport chain in the large mitochondrion (Vickerman,1985).

1.3. AFRICAN SLEEPING SICKNESS

1.3.1. The Disease

The *T.b.gambiense* and *T.b.rhodesiense* metacyclic trypanosomes enter the blood via the lymphatics then multiply and migrate to a wide variety of tissues and organs (Reviewed by

Poltera,1985). The widespread invasion by the trypanosomes throughout the host makes it difficult to treat the disease as some parts of the body are more inaccessible to a number of drugs than others (Gutteridge,1985). After several months to a year the trypanosomes can cross the blood-brain barrier and be detected in the cerebrospinal fluid. This entry into the brain can result in a diverse variety of symptoms ranging from a headache to a coma with death eventually following (Bentivoglio *et al*,1994).

1.3.2. Drug Treatment

The use of trypanocidal drugs to treat infected people is the most widely used method of controlling human trypanosomiasis. Only four drugs are used routinely in Africa to treat the disease; Suramin, Melarsoprol, Pentamidine and DFMO.

When the central nervous system is not infected with trypanosomes, Suramin is the drug of choice for treatment. It is effective against *T.b.brucei*, *T.b.rhodesiense* and *T.b.gambiense* but is ineffective against *T.congolense* and *T.vivax* (Hawking,1963). Suramin can distribute to most tissues but cannot cross the blood-brain barrier (Voogd *et al*,1993). This may explain relapse infections if trypanosomes can survive in organs or tissues which are not accessible by drugs. Suramin has a serum half life of 50 days and can act synergistically with DFMO (Clarkson *et al*,1984).

Melarsoprol is a melaminophenyl arsenical which, despite its toxicity causing death in 5-10% of treated patients, is the most widely used drug in treatment of sleeping sickness because it can cross the blood-brain barrier and is this effective against both acute and chronic infections. Melarsoprol has a serum half life of 35 hours and levels of the drug in the cerebrospinal fluid are 50-fold lower than that of the serum such that levels in the cerebrospinal fluid may be insufficient to cause lysis of some trypanosome strains (Burri *et al*,1993).

Pentamidine is a drug which is easier to administer compared to the others. It has in the past been used for large-scale prophylaxis and has limited side effects (Dukes,1984). Pentamidine cannot cross the blood-brain barrier.

DFMO is the only new drug to be developed for trypanosomiasis in the last 50 years. It blocks polyamine biosynthesis irreversibly thereby inhibiting trypanosome replication and enabling trypanosomes to be destroyed by trypanosome-specific antibodies (Metcalf *et al*,1978). DFMO treatment is expensive and large quantities of the drug must be administered which can result in physical limitations but there are only mild side effects. It is effective only against *T.b.gambiense*.

1.4. ANTIGENIC VARIATION IN *TRYPANOSOMA BRUCEI*

Antigenic variation is a classic evasion mechanism and can be seen in a number of pathogens including *Plasmodium falciparum*, *Neisseria gonorrhoeae*, *Candida albicans* and *Trypanosoma brucei* to escape humoral immune responses (Borst,1991a). In trypanosomes, antigenic variation is a specific mechanism by which the parasites, by changing their surface coat, evade the host immune response in order to prolong their infection. This process increases the probability of transmission to another mammalian host. The surface coat consists of a monolayer of a single molecular species of glycoprotein, the VSG, that physically protects the plasma membrane of the trypanosome from non-specific immune attack. Each VSG is antigenically distinct and determines the variable antigen type (VAT) of that parasite. An individual trypanosome thus expresses only one antigen on its surface but cells have the capacity to switch from expression of one VSG to that of another in the process of antigenic variation such that the population of trypanosomes which constitutes an infection contains many VATs.

The fluctuating parasitaemia is a classical characteristic of trypanosome infections. In the traditional view of an infection (see for example Vickerman,1985), a particular antigen type, called the 'homotype', forms the major part of the population at any one time and 99% of the

trypanosomes express the same VAT on their surface whereas the remaining 'heterotype' trypanosomes express different surface VATs. The host has the ability to clear the homotypic trypanosomes from the circulation with specific immune responses, but the remaining heterotypic trypanosomes continue to multiply and eventually one of these will become the homotypic population in the next parasitaemic wave until they are in turn destroyed leaving the way clear for subsequent heterotypic parasites and so on until death or cure (Borst & Greaves,1987). It has become clear in recent years that the traditional view is incorrect when dealing with fly-transmitted as opposed to syringe-passaged infections. Whilst some VATs are always more common than others at a particular time point in an infection, the concept of a homotype appears to be a laboratory artefact and populations routinely consist of several VATs at the same time (Barry & Turner,1991; Turner,1992; Barry,1997).

Within the VAT repertoire there appears to be a semi-predictable hierarchy of VAT expression. During infections there are several 'predominant' VATs that generally appear early in the infection and others that are expressed late in a sequence of expression which is partially predictable (Van Meirvenne *et al*,1975; Hajduck & Vickerman,1981; Miller & Turner,1981).

Reversible expression can occur in the trypanosome population and is an important feature distinguishing antigenic variation *sensu stricto* from antigenic diversity in the more general sense. This reversibility is usually detected by transferring trypanosomes from an existing chronic infection into a naive animal thereby "resetting" the VAT hierarchy of expression (Gray,1965; Van Meirvenne *et al*,1975). These VATs are not always in exactly the same order as they were seen in the original animal. Two VAT's may be switched in order or a VAT may be missed out altogether but the overall sequence is very similar.

It has been suggested that early in infection VATs with a high growth rate and a high switching frequency will be expressed and those with lower switching frequencies and/or growth rates will be seen later in infection (Capbern *et al*,1977; Myler *et al*,1985). The former will reach an undefined threshold to elicit an immune response which will lead to the

trypanosome destruction whereas the slower growing trypanosomes will not be at a sufficient level to induce an immune response and will therefore continue multiplying until they also reach a sufficient level. An increase in growth rate could be due to the life span of the stumpy forms decreasing, or by a decreased differentiation rate, or by an increase in replication rate of slender forms (Turner *et al*,1995). It should be noted that the evidence suggesting differences in growth rates between populations expressing different VATs (Diffley *et al*,1987) is weak (Aslam & Turner,1992) and it may be switching rate differences alone that are important in this context (Turner & Barry,1989).

The antigenic variation switching rate for a particular VAT may rely on either the 'switching on' rate or the 'switching off' rate or both (Turner & Barry,1989) with the success of antigenic variation depending on the VATs appearing in succession of each other instead of all at once (Barry & Turner,1991). It is not yet clear as to what stage in the cell cycle that switching occurs but it is cell cycle linked (Turner & Barry,1989). It has been shown that there is a large variation in switching rates, with low rate values of approximately 10^{-6} switches/cell/generation reported in syringe-passaged infections (Lamont *et al*,1986) but much higher switching rate of approximately 10^{-2} switches/cell/generation observed in fly-transmitted trypanosomes. The switching process is not induced by the host immune response but the rate of switching may be under selective pressure (Turner & Barry,1989).

All metacyclic VATs are also expressed during the initial early blood stage before switching to blood stage VATs although the control mechanisms for VAT expression in tsetse flies and mammals are independent of each other (Turner *et al*,1986). The metacyclic VATs are therefore a subset of the bloodstream VATs.

The VSG surface coat protects the parasites from the hosts non-specific immunity but is itself immunogenic and can therefore induce an immune response. Metacyclic trypanosomes and bloodstream, both slender and stumpy, trypanosomes are encapsulated by a VSG coat (Vickerman,1985). The VSG molecules are linked to the plasma membrane by glycosyl-phosphatidylinositol anchors (Cardoso de Almeida & Turner,1983; Ferguson *et al*,1988) with approximately 10^7 VSG molecules contributing to the bloodstream trypanosome coat

(Jackson *et al*,1985). Estimates of the quantity of VSG per trypanosome show reasonable agreement: 7.4mg VSG per 10^{10} trypanosomes (McGuire *et al*,1980), 7.6mg per 10^{10} organisms (Cross,1975) and 11.4mg per 10^{10} trypanosomes (Cardoso de Almeida & Turner,1983). Each VSG molecule consists of a variable and a constant domain defined by a trypsin cleavage site. The constant domain is towards the carboxyl terminus and contributes to approximately one third of the VSG whereas the remainder is the variable domain towards the amino terminus (Rice-Ficht *et al*,1981; Metcalf *et al*,1987; Carrington *et al*,1991). Only a fraction of the amino terminus is exposed to the host and therefore the host immune system, on live trypanosomes (Miller *et al*,1984; Gomes *et al*,1986). There are, very roughly, one thousand different VSGs (Van de Ploeg *et al*,1982) and each one generates a variable antigen type.

When a new VSG gene is switched on and the old VSG gene is switched off the old VSG surface coat is still expressed because it takes time before the old coat is completely removed and therefore there is a period when both old and new VSG are expressed together. These trypanosomes are called double expressors (Esser & Shoenbechler,1985). The VSG switching occurs gradually either by shedding of the VSG or by intracellular degradation. A high frequency of VSG uptake via the flagellar pocket and a relatively high amount of recycling was shown for the cytoplasmic pool of VSG but the degradation rate of VSG was low; only 2.2% of the VSG is shed per hour (Seyfang *et al*,1990).

T.brucei species vary in the number of 50-150kb mini chromosomes that they have. *T.b.gambiense* have approximately 10 mini chromosomes whereas *T.b.rhodesiense* and *T.b.brucei* have about 100 (Vickerman *et al*,1993). *T.congolense* has two but *T.vivax* and *T.equiperdum* have no mini chromosomes. The size and number of the intermediate sized chromosomes vary also in the range one to ten. It has been suggested that the smaller chromosomes are associated with antigenic variation due to the differences in intermediate and mini chromosome numbers between stocks and species and that the larger chromosomes represent the 'core' genome (Vickerman *et al*,1993; Erstfeld & Gull,1997). All chromosomes have VSG genes and they have two locations: at the telomeres or

intrachromosomal. The intrachromosomal loci are clustered into three arrays in the large chromosomes. Approximately 10% of the *Trypanosoma brucei* genome encodes VSG genes and VSG gene expression sites with a separate structural gene for each VSG and 10% of the trypanosomes biosynthesis is for production of VSG (Seyfang *et al*, 1990).

1.5. VACCINE DEVELOPMENT

In the case of African trypanosomiasis, vaccine development is extremely difficult. A cocktail of metacyclic VATs has been proposed as a vaccine for mammals but this could be unsuccessful for a number of reasons (Seed,1972): in some cases the serodemes are high in number, there are different serodemes depending on the geographical locality, and the metacyclic-VATs within a serodeme alter over a period of time. Apart from the VSG molecules, the procyclic surface molecules, transferrin receptors (Borst,1991b) and the flagellar pocket receptors (Olenick *et al*,1988; Shapiro,1994) have aroused interest as potential vaccine targets but as yet there have been no reports of successful, repeatable immunisation of animals based on these targets.

1.6. THE IMMUNE RESPONSE

1.6.1. The Humoral Response To African Trypanosomes

During African trypanosomiasis, chronically infected mammals have fluctuating parasitaemias, with successive parasitaemic waves, containing trypanosomes expressing different VATs, being successfully controlled each in turn by the humoral immune response. Seed & Sechelski (1987) compared normal intact mice with immunosuppressed mice and showed that the immunosuppressed mice showed a dramatic decrease in survival time and did not show a drop in parasitaemia whereas the intact control mice survived longer, produced a drop in parasitaemia and a second parasitaemic peak. Mice previously

immunised with irradiated *T.b.rhodesiense* can survive an infection with trypanosomes of homologous VAT, which would otherwise be deadly, but were killed if infected with trypanosomes of heterologous VAT. This resistance could not be transferred by T-cells but could be transferred by B-cells or serum to syngeneic recipients (Seed,1963; Campbell & Phillips,1976). The serum could transfer immunity for twice as long after the immunisation as the splenocytes. Taken together, these data show that VAT-specific humoral responses are protective but, due to antigenic variation, complete protection cannot be produced.

It is unclear whether the antibody responses generated in trypanosome infections are polyclonal or VAT-specific. There appears to be some degree of polyclonal B-cell activation in humans and domestic animals. The evidence for this is indirect and based on polyclonal hypergammaglobinemia shown by infected individuals. Diffley (1983) observed polyclonal activation of B-cells in trypanosome-infected mice leading to the depletion of antigen-reactive lymphocytes. Some polyclonal lymphocyte responses, which are associated with acute trypanosomiasis, were also observed when purified VSG was intravenously injected into mice (Diffley,1983). These observations could be associated with low affinity IgM binding non-specifically to antigens. Other authors however, have considered humoral responses against trypanosomes are almost entirely mediated by VAT-specific antibodies within the infected hosts and that mammals infected with a particular trypanosome VAT elicit a rapid humoral response against that VAT but not against trypanosomes of succeeding VATs (Van Meirvenne *et al*,1975; Musoke *et al*,1981). Musoke *et al* (1981) detected VAT-specific antibodies at the first antibody activity peak during *T.brucei* infections and also at the second peak of antibody response. This group failed to detect any non-specific antibodies at either of the peaks investigated.

The extant literature shows that both T-dependent and T-independent antibodies are detected during African trypanosome infections. Trypanosome-infected nude mice exhibit a decrease in their parasitaemia and this is due to the T-independent IgM antibody responses alone (Campbell *et al*,1978). These mice also showed an increase in their survival time compared to their heterozygous littermates. However, these infected nude mice did not

produce IgG antibodies, whereas the heterozygous mice did, suggesting that T-cells are required for antibody class switching. Another group found that *T.brucei*-infected nude mice exhibited lower B-cell responses in the absence of T-cells compared to immunocompetent mice (Clayton *et al*,1979). Others have shown that this humoral response is in the form of T-cell dependent IgM which is VAT-specific and that these responses control the fluctuating parasitaemia and result in the elimination of the trypanosomes that express that particular VAT (Van Meirvenne *et al*,1975; Musoke *et al*,1981; Black *et al*,1986).

As a generalisation, humoral responses against trypanosomes are characterised by having enhanced and prolonged IgM levels (Vickerman & Barry,1982). This contrasts with the typical textbook primary immune response of mammals where IgM production occurs transiently and is then replaced by IgG antibodies which have higher affinities and increased specificity compared with IgM antibodies. The IgM response fluctuates with the successive waves of parasitaemia and during parasite remission the IgM levels drop temporarily (Luckins,1976; Musoke *et al*,1981; Masake *et al*,1983) but this may be due to the antibodies being absorbed onto the surface of the trypanosomes and therefore being undetected during analyses of sera.

Increased serum IgM levels are found in trypanosome-infected humans, laboratory animals, cattle and sheep (Luckins,1976; Bouteille *et al*,1988). These increases can also be seen in animals infected with *T.congolense* or *T.vivax* (Luckins,1976). Masake *et al* (1981) revealed that during human sleeping sickness IgA and IgG levels remained the same as those from uninfected individuals whereas IgM levels were increased with a slight rise in IgE levels also. In mice, IgM, IgG_1, IgG_{2a} and IgG_{2b} isotypes were all produced in the humoral immune response but immunisation with irradiated trypanosomes was reported to stimulate an IgG response in preference to an IgM response (Diggs *et al*,1980; Sendashonga & Black,1982).

VAT-specific IgG production has been shown in *T.brucei*-infected cattle but has limited efficacy although specific IgG can be induced by immunisations (Musoke *et al*,1981).

Musoke *et al* (1981) have shown that trypanosome-specific IgM and IgG are produced in cattle during *T.brucei* infections which contrasts with the production of non-specific antibodies during murine infections. Cattle experimentally infected *T.congolense* or *T.vivax* showed both IgG_1 and IgG_2 levels changed during the infection with a 1-2 fold increase in both isotypes seen (Luckins,1976; Nielsen *et al*,1978; Bouteille *et al*,1988). In another study of *T.congolense* cattle infections, there were high levels of IgM, IgG_1 and IgG_2 antibodies produced against the infecting VATs and against successive VATs (Masake *et al*,1983). The IgG antibodies were detected seven days after the IgM response in cattle. Masake *et al* (1983) also noticed recurrent specific antibodies against VSGs and this could suggest that some VATs have similar surface coats or that there is a reappearance of certain VATs during the course of infection.

Antibodies have a role in the effector responses during trypanosomiasis. During cattle infections with *T.brucei*, the IgM produced at the first peak of antibody activity had increased efficiency for trypanosome neutralisation compared with IgG_1 antibodies but at the second antibody activity peak the opposite was found to be true, with all detectable antibodies being specific (Musoke *et al*,1981; Masake *et al*,1983). Macrophages were found not to phagocytose opsonised trypanosomes except in the presence of VAT-specific antibodies (Lumsden & Herbert,1967; Barry & Vickerman,1977). It is likely that the macrophage receptor for complement components mediates opsonisation *in vivo* as macrophages do not have Fc receptors for IgM. Destruction is due to VSG-specific antibodies and complement which leads to trypanosome lysis and phagocytosis by macrophages. Increased phagocytosis was also observed in the infected mice. There was an increase in hepatic clearance of trypanosomes in mice which were passively immunised with serum, irradiated trypanosomes or drug cured then reinfected (MacAskill *et al*,1980; MacAskill *et al*,1981) and this was due to the large number of kupfer cells present in the liver.

Most studies of responses to trypanosomes have focused on the bloodstream where most parasites are found, but, at least in human infections, most of the pathology is generated by

trypanosomes in extravascular sites. In this context it is important to note that IgM antibodies have been detected in cerebrospinal fluid and this detection is diagnostic for human sleeping sickness (Mattern,1962). Goodwin & Guy (1973) also detected trypanosome-specific antibodies in *T.brucei*-infected rabbit tissue fluid two days later than in serum and at reduced titres.

Immunity can be transferred to young mice from mothers if the young mice suckle from birth. This immunity was transferred in the mothers colostrum/milk if the mother was infected with *T.brucei* or if the mother had been drug-cured before giving birth (Whitelaw & Urquhart,1985). This immunity was not transplacental, as young mice born from infected mothers were not immune to trypanosome infections if they suckled on normal uninfected mothers from birth. These data are therefore suggestive of a protective IgA response. Takayanagi *et al* (1978) showed that neonatal rats could also be protected from *T.b.gambiense* infections if they suckled from the mother which gave birth to them. In rats however, if the neonates received colostrum by suckling from normal mothers then they were found to be susceptible to trypanosome infections which suggested an *in utero* component was essential for transferring immunity from mothers to neonates. These authors concluded that the IgA present in the mothers colostrum was not effective at protecting the young against trypanosome infections compared with the IgG obtained via the placenta.

1.6.2. The Complement System During African Trypanosomiasis

During African trypanosome infections, the parasitaemic waves are followed by lysis of parasites on a large scale due to the production of VSG-specific antibodies and the complement cascade (Balber *et al*,1979). It is not clear however which complement pathway is involved in trypanosome lysis. It has been suggested that the classical pathway can be activated by VSG interactions with specific antibodies and that the alternative pathway can be initiated by an antibody-related distortion of the VSG thereby exposing the

plasma membrane (Ferrante & Allison,1983). This infers that if the trypanosome VSG remains intact then the alternative pathway will not be activated.

Ethylenediamine tetraacetic acid (EDTA), by blocking the classical pathway, inhibits lysis of *T.brucei* and *T.congolense* whereas ethyleneglycol-bis-(b-amino-ethyl ether) N',N'-tetraacetic acid (EGTA) did not (Balber *et al*,1979; Ferrante & Allison,1983) and these data, together with the requirement for specific antibodies, suggests that it is likely that the classical pathway is of greater importance. There is contrasting evidence for involvement of the alternative pathway: in the presence of EGTA or in C4 deficient serum *T.b.rhodesiense* is lysed (Flemming & Diggs,1978). Flemming & Diggs (1978) suggested that the alternative complement pathway is responsible for antibody-dependent cytotoxicity against *T.b.rhodesiense* due to the dependence on magnesium ions and there is no need for calcium ions. EDTA prevented immune cytotoxicity for trypanosomes and EGTA had very little effect on anti-trypanosome cytotoxic reactions implying that the alternative pathway was responsible for lysis. C1 activation may be inhibited by calcium ion chelation by EGTA which, when restored, could reinstate the lytic function but not the cytotoxic function (Flemming & Diggs,1978; Ferrante & Allison,1983).

VAT-specific antibodies are important in the opsonisation and lysis, in the presence of complement, of trypanosomes with stumpy forms being more resistant to lysis than slender *T.brucei* forms expressing the same VAT (Barry & Vickerman,1977). Ferrante & Allison (1983) have stated that specific murine IgM does not fix mouse complement and therefore fails to activate the alternative pathway. This may contribute to the high susceptibility of murine African trypanosomiasis as IgM appears to be the first line of the hosts defence against this parasite. In contrast, however, Diggs *et al* (1980) claim that IgM, IgG_1, IgG_{2a} and IgG_{2b} mouse antibodies against *T.b.rhodesiense* are all capable of causing trypanosome lysis by activating the alternative pathway.

1.6.3. African Trypanosomiasis And Cell Mediated Immunity

There is an increase in the activity and size of germinal centres in the spleen and lymph nodes of Boran cattle infected with *T.vivax* as well as an increase in the number of large proliferating lymphocytes and plasma cells (Masake & Morrison,1981). These authors stated that the increase in spleen size was due to the white pulp areas expanding and taking over the areas which were previously red pulp areas whereas in the lymph nodes it is the medullary region which increases. During trypanosome infections in cattle the spleen reaches a maximum weight and then returns to a normal size later on in the infection and this reduction is associated with a decrease in the number of red pulp plasma cells and a reduction in the white pulp size and activity. The increase in the lymph node size occurs several days after that of the spleen and it is predominantly the responsibility of the lymphocytes, especially large proliferating lymphocytes (Masake & Morrison,1981). This is only to be expected as trypanosomes are parasites of the blood such that it seems reasonable to assume that this would be the primary site of the immune response. It would only be when the trypanosomes enter the lymph fluid the increase in lymph node size occurs, slightly later in the infection. Masake & Morrison (1981) showed no significant proliferative differences between white blood cells of control and *T.vivax*-infected cattle to mitogens which were used *in vitro*. During murine trypanosome infections the lymphoid cells, macrophages and null cells proliferate in the lymphoid organs which in turn increase in size (Askonas & Bancroft,1984). Splenomegaly is also a prominent feature during trypanosome infections in mice (Murray,1979; Murray & Morrison,1979; Jenkins & Facer,1984). The spleens of *T.rhodesiense*-infected mice were seen to become heavily infiltrated with macrophages and plasma cells during infection.

There are T-cell independent and T-cell dependent VSG-specific responses during trypanosome infections (Mansfield,1994) and these responses depend on the way in which the VSG coat is recognised by the host immune system. When the VSG is on the trypanosome, it is a repetitive 3-dimensional structure with epitopes which can be easily

recognised by antibodies. Antibodies recognise tertiary protein structures and this response is classed as T-cell independent. When the VSG is shed or broken down it forms monomers that may loose their structural integrity and do not have repetitive epitopes. These monomers can be more easily presented to T_H cells which will lead to a T-cell dependent response. The evidence for a T-cell dependent response is highly variable in character in that trypanosome-infected nude mice have decreased B-cell responses compared with fully competent mice (Clayton *et al*,1979) and these infected nude mice cannot mount an IgG response without T-cells present (Campbell *et al*,1978). Also, irradiation and reconstitution of chimeric mice during trypanosome studies (DeGee & Mansfield,1984) and lack of relationship of longevity of infected mice with a quality antibody response (Sendashonga & Black,1982; Bouteille *et al*,1988) suggests an important role for T-cells during trypanosome infections.

Schleifer *et al* (1993) did not detect proliferation of T-cells in any of the lymphoid tissues when they stimulated with purified VSG or with whole cell extracts using mice which had been trypanosome-infected for two weeks. At this time point there was some response to Concanavalin A (Con A) in the lymph nodes but no response from the peritoneal cells or spleen cells. This lack of, or very limited response to, Con A is probably due to the immunosuppression induced by two weeks of infection. This group also demonstrated that there were different cytokine responses in different compartments of the host. They identified VSG-specific T-cells in the peritoneal cavity and although these cells did not proliferate they did produce cytokines when stimulated with VSG *in vitro*. Substantial amounts of IL-2 and IFNγ were produced by peritoneal lymphocytes which is typical of a T_H1-type response. However T_H1 and T_H2 cells, which are VSG-specific, are produced after immunising with VSG. These authors also state that a population of VSG-specific T-cells which produce IFNγ were "sometimes" found in the spleens of infected mice but they do not elaborate further about when during the infection that these cells secrete IFNγ. The lack of a proliferative response could suggest that there is a lack of costimulation between the T-cells and macrophages which prevents proliferation. It has been suggested that

macrophage activation during trypanosome infections is not due to the phagocytosis of bloodstream trypanosomes and that the mechanism, as yet, remains unknown (Rossi *et al*,1987). Primed T-cells have the ability to increase the macrophage response even in the absence of trypanosomes but the response can be further increased in the presence of trypanosomes. These results therefore suggest that T-cells may be important in the regulation of macrophage function during trypanosomiasis.

Complement, Fc and mannose receptors on the surface of peritoneal macrophages decrease during *T.b.brucei* murine infections which therefore affects the phagocytosis and degradation of trypanosomes by the macrophages. During infection, resident spleen and peritoneal macrophages, as well as recruited macrophages, alter in appearance (Askonas & Bancroft,1984). Macrophages also have the ability to suppress or enhance antibody responses *in vivo* which are T-cell dependent. Immune complexes, formed by the trypanosomes, antibody and/or complement, can have different fates depending on the size of the complexes and these can be phagocytosed by macrophages or they can induce immunosuppression.

Splenic mononuclear cells and peripheral blood mononuclear cells from *T.congolense* infected cattle proliferated *in vitro* to homologous and heterologous trypanosome challenge although this was not the case in the lymph node tissues (Flynn *et al*,1992). *In vitro* T-cell proliferation was not shown in the lymph nodes but occurred in the peripheral blood and the spleen although the trypanosome surface coat did not induce this proliferation.

One group has focused on CD8 cells in trypanosome infections rather than CD4 cells. Bakhiet *et al* (1993a) claim that *T.b.brucei* parasites, during infections of rats and mice, release a 'lymphocyte-triggering factor' which binds to and activates $CD8^+$ lymphocytes and that $CD4^+$ cells are not activated. This activation results in the production of IFNγ and Transforming Growth Factor-β (TGFβ), which induces immunosuppression. In *T.b.brucei*-infected rats, IFNγ production was greatly reduced when the CD8 T-cells were depleted *in vivo* and this resulted in an increased survival time of the rats (Bakhiet *et al*,1990). No clinical signs of disease were seen in the infected rats which were CD8 depleted until four

weeks after they had been infected and at this point the blood parasitaemia increased but these treated rats out-lived the control rats by three weeks. Mononuclear cells from mice lacking CD8 expression did not produce IFNγ when stimulated with the 'lymphocyte-triggering factor' *in vitro* but did produce IFNγ when stimulated with Con A and the parasitaemias of $CD8^+$ mice were found to be approximately 10-fold higher than those in CD8 knockout mice (Olsson *et al*,1993). Olsson *et al* (1991) claim that CD8 lymphocytes have to be activated in order to achieve IFNγ production during trypanosome infections but parallel studies of T_H1 CD4 T-cell or NK cell involvement demonstrating that they do not produce IFNγ have not been made by this group.

1.7. IMMUNOSUPPRESSION IN TRYPANOSOME INFECTIONS

Different African trypanosome strains differ in virulence and growth rate within different mammalian hosts therefore differing in the degrees of immunosuppression that they generate (Sacks *et al*,1980). Mammalian hosts infected with African trypanosomes are in a state of generalised immunosuppression (Flynn & Sileghem,1991; Murray *et al*,1974) which has some serious repercussions for the host, including increased susceptibility to secondary infections and a continuous high parasite load. Host antibody and T-cell responses to infecting trypanosomes, other invading pathogens and to mitogenic stimulus can be immunosuppressed during trypanosomiasis although in some cases it is only the proliferative responses which appear to be affected (Dempsy & Mansfield,1993; Gasbarre *et al*,1981; Vickerman & Barry,1982).

1.7.1. Depression Of Humoral Responses

T.brucei-infected laboratory animals have impaired antibody responses to sheep red blood cells (RBCs) with immunosuppressed antibody responses also occurring in cattle and humans (Goodwin,1970). The IgG responses are suppressed more quickly than the IgM

responses and to a greater extent (Hudson *et al*,1976). The suppressed host response to sheep RBCs is somewhat irrelevant, in my opinion, as the chances of these blood cells causing considerable harm or death to the host are negligible and therefore the hosts response is unimportant.

What is more important is the depression of trypanosome-specific responses. Both IgM and IgG antibody responses are decreased, compared with uninfected controls, at the first peak of parasitaemia during acute *T.brucei* infections and during chronic infections (Masake *et al*,1983). There is also decrease in IgM and IgG responses at each parasitaemic peak in contrast with the previous peak, but with sufficient amounts of IgM still available to control the infection. The trypanosomes can suppress IgM and IgG responses to both the trypanosomes and unrelated antigens (Sacks & Askonas,1980) although others (Hudson *et al*,1976) have suggested that IgM levels appear to be trypanosome-specific and remain at high levels when other responses are immunosuppressed. The level of parasitaemia correlates with the degree of IgM suppression (Sacks *et al*,1980).

1.7.2. Cell Mediated Immunodepression

There is general agreement that a pronounced depression of immune responses to bystander antigens accompanies trypanosome infections (reviewed in Askonas,1985) and this suggestion extends to T-cell proliferative responses against trypanosome antigens (Gasbarre *et al*,1980). It should be noted however that one author, Freeman *et al* (1974), could not detect any suppression of cell mediated immunity.

A number of mechanisms of immunosuppression have been proposed for trypanosome infected hosts, but with a consensus that macrophages are strong effectors at inducing suppression (Askonas,1985; Borowy *et al*,1990; Flynn & Sileghem,1991) with splenic and peritoneal macrophages found in increased numbers during trypanosome infections due to the recruitment of cells into these areas (Askonas,1985).

Splenic immunosuppression occurs several weeks before that of the lymph nodes with the spleen being the organ most affected during trypanosome infections (Kar *et al*,1981; Wellhausen & Mansfield,1980). Spleen cells from trypanosome-infected mice can decrease the proliferation of uninfected mice splenocytes when they are cultured together *in vitro* (Borowy *et al*,1990).

The evidence implicating macrophages in immunosuppression is very strong. A mixed culture of macrophages from uninfected and infected mice resulted in the proliferation of T-cells with no significant suppression of responses but if there were no macrophages from the uninfected mice then T-cell proliferation to the trypanosomes did not occur (Gasbarre *et al*,1981; Borowy *et al*,1990). If infected mice were drug cured then their cells were able to respond as those of normal uninfected mice with a normal level of responsiveness three weeks after drug cure (Gasbarre *et al*,1981).

Macrophage activation appears to be important in immunosuppression due to the release of mediators, cytokines and the alteration of cell surface markers when the opsonised trypanosomes are phagocytosed. IFNα, IFNβ, IFNγ, IL-1, IL-2, prostaglandin E_1, prostaglandin E_2 and prostaglandin F_2 are produced by activated macrophages but they have no direct effect on the trypanosomes (Vickerman *et al*,1993) with macrophages from infected mice producing far more prostaglandin E_2 than postacyclin and vice versa in control mice (Askonas,1985). During trypanosomiasis, NO is released from macrophages due to the synergistic effect of IFNγ and TNFα (tumour necrosis factor α) whereas IL-4, IL-10 and TGFβ downregulate IFNγ production and thereby prevent nitric oxide (NO) production by the activated macrophages (Vincendeau *et al*,1992). In mice, the macrophage signalling network must be complete to allow trypanosome infections to survive. Mice with naturally occurring immunodeficiences in the macrophage population caused a decrease in trypanosome survival time by 50% whereas deficiencies in complement levels or NK cell activity did not affect trypanosome survival (Jones & Hancock,1983). These experiments showed that Beige mice (lacking NK cells) actually lived longer than control mice.

In cattle, when macrophages were depleted from the lymph node populations of *T.congolense*-infected Boran cattle the immunosuppression effect was also removed (Flynn & Sileghem,1991). In contrast to the prevailing view that macrophages essentially mediate suppression, Jayawardenna *et al* (1978) have argued that it is the T-cells which induced suppression of both the macrophages and the antibody response. Whether macrophages or T-cells initiate suppression could be a circular argument because the macrophages require IFNγ to function but the T-cells are being prevented from producing the IFNγ by the macrophages either directly or indirectly. This argument ignores the potential role of NK cells that can produce large amounts of IFNγ which would potentially stimulate macrophages. It is possible that the factor which prevents the macrophages from functioning also affects the NK cells or alternatively that IFNγ is not critical to the cell mediated response against African trypanosomes. Another explanation might be that IFNγ is important but during infection is produced in such high amounts that a state of general immunosuppression is induced and therefore the T-cells and macrophages cannot function. It has been shown by Schleifer *et al* (1993) that IFNγ-activated macrophages from infected mice can produce prostaglandins and NO which in turn can interfere with T-cell proliferation which suggests that macrophages may control the cellular immune response.

Macrophages from infected mice can inhibit lymphocyte responses which are linked with IL-2 receptor expression and IL-2 release (Kierszenbaun *et al*,1991; Sileghem *et al*,1986; Sileghem *et al*,1989). Peripheral blood mononuclear cells show decreased IL-2 receptor expression with levels of IL-2 unaffected during *T.b.rhodesiense* infection (Kierzenbaun *et al*,1991). This was only the case when live trypanosomes were used, that is, there was no decrease in IL-2 receptor expression when the parasites were gluteraldehyde-fixed. IL-2 receptor expression levels could be restored in lymph node cells of *T.brucei*-infected mice by administering anti-IFNγ antibodies but this was not the case with the splenic population (Darji *et al*,1993). In the lymph node population of *T.brucei*-infected mice, the IL-2 levels were decreased and this was due to macrophages inhibiting its production in a prostaglandin-dependent mechanism (Darji *et al*,1993; Sileghem *et al*,1989; Sileghem *et al*,

1991). IL-2 receptor expression was also inhibited in a prostaglandin-independent manner and was affected on $CD4^+$ and $CD8^+$ cells alike. Using Con A as a stimulant of lymph node lymphocytes from a murine *T.brucei* infection there appeared to be an inhibition of IL-2 production and this inhibition was not related to a lack of accessory cells or functioning accessory cells (Sileghem *et al*,1986). Exogenous IL-2 restored the activity of the lymph node cells which were stimulated with Con A (Sileghem *et al*,1986). This inhibition may be related to the control of immunosuppression by inducing anergy if these cells exhibit IL-2 receptors on their surface during trypanosomiasis. Schleifer *et al* (1993) however produced evidence that substantial amounts of IL-2 and IFNγ were produced by peritoneal lymphocytes which is indicative of a T_H1-type response.

The general consensus is that cell-mediated immunosuppression does occur during African trypanosome infections with both the $CD4^+$ and $CD8^+$ T-cell populations suppressed and a lack of IL-2 production. Macrophages are the major effector cells which have an important pivitol role in inducing this suppression thereby allowing the parasite to continue infection.

1.7.3. Effects Of Nitric Oxide During African Trypanosome Infections

NO is a major product of activated macrophages with cytostatic and cytotoxic properties and thus merits particular attention in the context of a trypanosome infection. NO can directly inhibit trypanosome proliferation *in vitro* (Sternberg *et al*,1994; Vincendeau *et al*,1992). Since S-nitroso-acetyl-penicillamine (SNAP), a NO donor, inhibits trypanosome proliferation but N^G-nitro-L-arginine methyl ester (L-NAME) restores this proliferative function it suggests that NO has a cytostatic, rather than a cytotoxic, effect on trypanosome growth *in vitro* (Sternberg *et al*,1994). Oxyhaemoglobin is a NO scavenger so it is therefore unlikely that NO will have any direct effects on trypanosomes in the bloodstream of the host (Mabbott *et al*,1994). Nevertheless, prolonged survival and lower parasitaemias were

observed in mice which were infected with NO-treated parasites compared to control parasites suggesting that NO may have an indirect affect (Vincendeau *et al*,1992).

The NO produced by infected mice peritoneal and splenic macrophages is important in the specific immunity of the host. In general, suppression and inhibition of splenic T cell proliferation is due to the release of reactive nitrogen intermediates (RNI) from the NO synthase pathway (Mills,1991). This link between suppression and NO has been observed in mice infected with *T.brucei* (Borowy *et al*,1990; Mills,1991; Sternberg & McGuigan,1992). N^G-monomethyl-L-arginine (L-NMMA), a specific inhibitor of the inducible NO synthase pathway, substantially reduced the accumulation of nitrite in cell cultures and could prevent the cytostatic activity that the activated macrophages induce, strongly suggesting that the RNI were produced via the L-arginine dependent pathway of NO synthase (Mills,1991; Sternberg & McGuigan,1992; Vincendeau *et al*,1992). The amounts of NO produced from cells of *T.b.rhodesiense* infected mice increased after stimulation with specific parasite antigens or T-cell mitogens and the suppression induced by macrophages from infected mice could be prevented if NO and prostaglandins were inhibited together (Schleifer & Mansfield,1993).

In conclusion, immunosuppression during African trypanosomiasis could be due to several mechanisms induced by the trypanosomes to inhibit the host T-cells from providing help for the B-cells. The inhibition of T-cell functions, perhaps by NO, could be a way of preventing the host from producing a more specific and more potent IgG response which would have a higher affinity than the IgM response. The IgM response is not substantially affected by the immunosuppression whereas the IgG response, which strongly depends on T-cell help, can be suppressed during infection.

1.8. AIMS OF THE PROJECT

The aims of my project were to investigate the possibility of antigenic variation as a

T-cell evasion mechanism during *Trypanosoma brucei* infections and, if this was found to be the case, determine whether these T-cells could drive this antigenic variation. Clearly, it was also important to establish if T-cells played any significant role in determining the course of infection and the mechanism(s) by which T-cell responses might be regulated. More specifically, this study involved:

1. Designing a reliable *in vitro* assay system to examine T-cell proliferative responses towards a number of different trypanosome VATs;
2. Examining a number of different trypanosome infection and immunisation regimes;
3. Predicting potential T-cell epitopes in the VSG trypanosome coat and investigating their ability to provide protection;
4. Investigating the role of NO in the control of T-cell responses.

CHAPTER 2

MATERIALS AND METHODS

2.1. ANIMALS

Young adult female CFLP (25-30g) mice were intraperitoneally (i.p.) inoculated for growth of trypanosomes to provide parasite materials for use in other experiments. When the infection was the subject of study in itself, young adult female BALB/c (20-25g) mice were used by i.p. inoculating 10^4 trypanosomes/mouse from the infected CFLP blood. When immunising mice with parasites, Balb/c mice were i.p. injected with 10^6 paraformaldehyde-fixed trypanosomes/mouse in Phosphate Buffered Saline (PBS), pH 7.4. The mice were left for 2 weeks before killing or carrying out further procedures.

Outbred adult female MF1 (25-30g) mice were used for experiments in Chapter 7. Mice homozygous and lacking a functional inducible nitric oxide synthase (iNOS) gene locus (Wei *et al*,1996) were i.p. injected with 10^4 trypanosomes/mouse from infected CFLP blood. MF1 mice heterozygous for the iNOS gene were used as controls. These mice were a kind gift from Prof. F.Y. Liew, Department of Immunology, University of Glasgow.

Female WISTAR rats (200-250g) were used for growing larger numbers of trypanosomes required for absorption assays. 1ml of trypanosome-infected blood, exsanginated blood from infected CFLPs, were injected via an i.p. route into each rat.

2.2. TRYPANOSOMES

Stabilates, from a liquid nitrogen store, were thawed and taken up into a syringe of 0.5ml Carter's balanced salt solution (CBSS) containing 100U/ml Heparin for injection into mice. The monomorphic *T.brucei* clones, which each stably express a single VAT at the first wave of parasitaemia, were ILTat (International Laboratory for Research on Animal Diseases, *Trypanozoon* antigen type) 1.3, ILTat 1.61, GUTat (Glasgow

University *Trypanozoon* antigen type) 7.1, GUTat 7.2 and ANTat (Antwerp *Trypanozoon* antigen type) 1.8 [Figure 2.1a-d]. For the chronic infections, a GUTat 7.2 pleomorphic trypanosome line was used [Figure 2.1e.]. ANTat 1.8 (GUP 3265 and GUP 4284) is a subclone expressing a single VAT from the cloned stock EATRO 1125. All VATs used in this project are serologically distinct from each other (Van Meirvenne *et al*,1975; Turner, unpublished results). These six lines of trypanosomes were used throughout this study in infections, in a paraformaldehyde-fixed state for immunisations and as an antigen source in assays.

2.3. TRYPANOSOME PURIFICATION

Two methods were used. For small scale preparation of trypanosomes, a differential centrifugation method similar to that of Ghiotto *et al* (1979) was employed. Blood was removed from anaesthetised mice by cardiac puncture into a syringe containing 0.2mls of CBSS/Heparin. The blood sample was transferred into a 15ml centrifuge tube and mixed with 2 volumes of PBS pH 7.4. The tube was centrifuged for 7 minutes at 200×g. The top straw coloured layer was removed to a fresh tube and spun at 2600×g for 5 minutes. This pelleted the trypanosomes which were resuspended in 1ml of PBS pH 7.4, counted using an Improved Neubauer haemocytometer and resuspended at the required density.

When the blood was removed from the infected rats it was exsanguinated into a syringe containing 1ml CBSS/Heparin, then mixed with Percoll (Pharmicia, Sweden) containing 8.55g sucrose (BDH), 2g glucose (Sigma) and the pH adjusted to 7.4 with solid HEPES (Sigma) as described by Grab & Bwayo (1982) and RPMI 1640 (Dutch modification) medium (Gibco) at a ratio of 1:5:2, respectively. The samples were centrifuged at 4°C for 20 minutes at 38000×g. The layer of trypanosomes was removed to a fresh tube for counting.

Populations were checked for VAT homogeneity by immunofluorescence on air-dried smears fixed in 70% ethanol.

2.4. IMMUNOFLUORESCENCE ANTIBODY TECHNIQUE

The trypanosome smears were prepared, air dried and fixed in 70% ethanol for 1 hour at room temperature 4°C. They were then used immediately or stored at -20°C in the presence of silica gel. After return to room temperature if required, the reaction zones were drawn onto the slides using plastic ink (Texpen, Mark-Tex Corporation, Englewood, New Jersey). Once the ink had dried, the slides were rehydrated in 10% FCS in PBS for 30 minutes in a humid chamber. The slides were then washed twice in 0.1% Polyoxyethylene-sorbitan Monolaurate (Tween 20)/PBS (v/v). An appropriately diluted first antibody preparation [Tables 2.1a-d.], in PBS/Tween 20, was applied to the reaction zones and slides were incubated in a humid chamber for 30 minutes at room temperature. All VAT-specific antibodies were provided by C.M.R. Turner. After incubation, the slides were tapped on the bench to remove most antibody solution and the slides were washed for 5 minutes twice by submerging in Coplin jars filled with PBS/Tween wash buffer. Excess PBS was removed from outside the reaction zones using a tissue. Care was taken to ensure reaction zones did not dry out. The appropriately diluted conjugated secondary antibody [Table 2.1a-d.] was then applied to the reaction zones. All secondary antibody preparations were diluted in PBS/Tween 20 with 4',6-Diamidino-2-phenylindole (DAPI) being used as a counterstain for DNA material (0.01mg/ml), prepared by a 1 in 50 dilution of a stock solution stored for up to 3 months at 4°C. The slides were incubated in a humid chamber for 15 minutes at room temperature. All secondary antibodies were titrated for optimal fluorescence activity before use. The slides were washed briefly in PBS/Tween 20 twice before mounting the coverslip with Citifluor antifadent (Citifluor Ltd). The trypanosomes were then examined by immunofluorescence microscopy to ensure a homogeneous population was present.

Immunofluorescence was also carried out on live trypanosomes purified as previously described (Section 2.3.). 10^6 trypanosomes/100µl PBS pH 7.4 were aliquoted on ice. The staining process was the same as for the fixed trypanosomes, with the exception of the washes which were with PBS pH 7.4, as were the antibodies and their dilutions

[Table 2.1a-d.]. Mouse monoclonal 23.2, for a subsurface epitope of ILTat 1.3, and mouse monoclonal 17.2, a subsurface antibody for GUTat 7.1, were used as neat hybridoma culture supernatants with the live trypanosomes with 50µl/aliquot and appropriate controls. The trypanosomes were examined by immunofluorescence microscopy.

2.5. PARAFORMALDEHYDE FIXATION OF TRYPANOSOMES AND SHEEP RED BLOOD CELLS

Trypanosomes expressing a single antigen type were purified from infected mouse blood as described previously (Section 2.3.). Populations were checked for antigenic homogeneity by immunofluorescence (Section 2.4.). Purified trypanosomes, resuspended in 1ml of PBS pH 7.4, were incubated at room temperature for 20 minutes with 1ml of 4% paraformaldehyde in 0.1M PBS. The trypanosomes were then washed 3 times in PSG (PBS containing 1% glucose). The trypanosomes were, after the final 5 minute centrifugation at 2600×g, left overnight at 4°C in 0.1M ammonium chloride in 0.1M PBS to neutralise any residual NH_2 groups. The trypanosomes were centrifuged, as before, and resuspended in PBS pH 7.4 at a concentration of 10^7 trypanosomes/ml. Immunofluorescence was also performed on smears of these fixed parasites to check that the antigenic identity had been preserved after the fixing process (Section 2.4.).

Sheep RBCs (SAPU, Scotland) were also paraformaldehyde-fixed as described above for the trypanosomes. The sheep RBCs were isolated from blood by centrifugation, counted and resuspended in RPMI 1640 supplemented medium containing 2mM L-Glutamine (Sigma), 0.75% sodium bicarbonate (BDH), 2% gentamycin (Sigma) and 10% FCS (Gibco) at 10^7/ml and dispensed at 5×10^5/well.

2.6. ISOLATION OF MONONUCLEAR SPLENOCYTES

All reagents and equipment were sterilised before use. The medium used was RPMI 1640. Spleens were removed from mice, mashed through metal mesh (Tea strainers,

Woolworths) into 10mls of supplemented RPMI 1640 medium, in the case of uninfected spleens, and into 30mls when the spleens were from infected or immunised mice. The cells were passed through monofilament nylon filter cloth (R.Cadisch & Sons, London), 100 μm, to obtain a single cell suspension. Cymelarsan (Rhone Merieux, France) was added to each suspension at 50μg/ml to kill trypanosomes and suspensions were monitored by microscopy until lysis of trypanosomes was observed (usually 10-30 minutes). The splenocytes were centrifuged at 200×g for 7 minutes, the supernatant decanted and the loose pellet resuspended in 5mls if from uninfected mice, or 20mls for suspensions from infected mice, of supplemented RPMI 1640 medium. The suspensions were layered onto 3ml cushions of Nycoprep (Nycomed UK Ltd, Birmingham) and centrifuged for 15 minutes at 700×g. The interface layer was removed, centrifuged for 7 minutes at 200×g and the pellet was resuspended in 2mls medium. The phase bright cells were counted using an Improved Neubauer haemocytometer and resuspended at the correct density. Preliminary experiments using Trypan Blue exclusion revealed greater than 95% viability when purifying these cells.

2.7. PERITONEAL CELL EXTRACTION

Balb/c mice were euthanised, swabbed with 70% ethanol and a ventral mid-line incision made in the skin over the peritoneum. 5mls of RPMI 1640 medium was i.p. injected with a 21G needle above the liver. The abdomen was massaged to dislodge adherent cells and the skin was peeled back. The needle was reinserted below the splenic pocket, cells removed and transferred to a 50ml falcon tube. Cymelarsan was added to lyse the trypanosomes present and the cells were separated as before (Section 2.6.). When the cells were separated, smears were made for Giemsa staining to count the numbers of lymphocytes and macrophages present.

2.8. PROLIFERATION ASSAYS

The isolated cells were plated out at $2x10^5$ cells/well in sterile flat bottomed 96-well microtitre plates (Greiner). Concanavalin A (C-5275, Sigma), at 8μg/ml, was the positive control with medium alone as the negative control. The fixed trypanosome VATs were used as the antigen source at 2×10^6 trypanosomes/ml. All wells were made up to the same volume using supplemented RPMI 1640. The cells were incubated at 37°C with 5% CO_2 in a humidified incubator. After 48 hours the cells were radiolabelled with 1μCi/well of ^{3}H-Thymidine (Amersham International) and harvested 16 hours later onto fibreglass filtermats and read on a Betaplate counter (Becton Dickinson). The proliferative responses were determined by ^{3}H-Thymidine incorporation and results are expressed as the mean counts per minute of four wells ± 2 SE.

2.9. CYTOKINE ASSAYS

The mononuclear splenocytes were dispensed into sterile flat bottomed 24-well plates (Greiner) at $4x10^6$ cells/well and the peritoneal cells were plated at 1×10^6 cells/well. Medium alone was used as a negative control with Concanavalin A (Con A) as the positive control at 8μg/ml. Fixed trypanosomes expressing specified VATs were used as the antigen source at a concentration of $2x10^6$ trypanosomes/ml. In total, each well was made up to a final volume of 1.25mls with medium. The plates were incubated under the same conditions as the proliferation assays and the supernatants were harvested at 24 and 72 hours. The supernatants were pulsed in a microfuge at 10000×g to pellet the cells and the cell-free supernatants were transferred to fresh tubes and stored frozen until analysed by ELISA (Enzyme Linked Immunosorbant Assays).

The harvested supernatants from the cytokine assays were examined for IL-2, IL-4, IL-5, IL-6 and IFNγ using commercial capture and detection antibodies (Pharmingen, Cambridge Bioscience). The capture antibodies were diluted in 0.1M sodium hydrogen carbonate buffer pH 8.2 and added to wells of Immulon 4 96-well high affinity binding plates (Dynatech). The plates were incubated at 4°C overnight. The plates were washed

twice with 0.01M PBS/Tween 20 (0.05% v/v) and blocked with 10% FCS/PBS (v/v) for 1 hour at 37°C. The plates were washed twice with PBS/Tween 20 and the relevant standards (Pharmingen) and samples added in triplicate. Each plate assayed contained a standard curve. The plates were incubated for 3 hours at 37°C, washed 4 times and the detection antibodies, diluted in 10% FCS/PBS, added. The plates were further incubated at 37°C for 1 hour then washed 6 times before adding the extravadin peroxidase (SAPU), diluted at 2μg/ml in 10% FCS/PBS. After the final 1 hour incubation and 8 washes, 100μl/well of the TMB Microwell peroxidase substrate (Kirkegaard & Perry, Dynatech) was added and the colour allowed to develop (5-10 minutes) at room temperature. Plates were all read immediately at 630nm, with a reference filter of 405nm as instructed using Biolinx software (Dynatech). The results are expressed as the mean of triplicate wells minus the mean background levels, that is the readings obtained from the wells without any sample.

2.10. FLOW CYTOMETRY ANALYSES

The cells were separated as previously described (Section 2.6.). Aliquots of 10^6 cells were made and resuspended in 50μl of 5% FCS/PBS/0.05% azide wash buffer. The cells were incubated with 3μl/aliquot of antibody and incubated on ice for 1 hour. Commercial antibodies (Pharmingen, Cambridge Bioscience) were used to detect CD3, CD4, CD8, CD19, surface Ig, NK 1.1 and CD25 (to measure IL-2 receptor expression). The cells were washed twice in FCS/PBS and the secondary antibodies added, if required, and the cells incubated for a further hour. The cells were washed twice then resuspended in 200μl 0.15M Tris ammonium chloride pH 7.3 for 10 minutes to remove any contaminating RBCs. The cells were microfuged briefly at top speed (10000×g) and resuspended in PBS pH 7.0 before analysis on the FACScan flow cytometer (Becton Dickinson). The lymphocyte population was gated and 5000 cells counted. Some experiments resulted in fixing the cells with 1% paraformaldehyde in PBS, pH 7.0, and analysing 24 hours after staining. There appeared to be no difference in the results when comparing the staining of the fixed cells with those resulting from unfixed cells. All the

results presented in this thesis are from unfixed preparations. Early experiments used biotinylated anti-CD3 and biotinylated anti-NK 1.1 antibodies and then a secondary antibody of Streptavadin-FITC (Fluorescein isothiocyanate). Later experiments used a directly conjugated phycoerythrin (PE) anti-CD3 antibody. All other antibodies were directly conjugated: PE anti-CD4; PE anti-CD8; FITC anti-CD8; FITC anti-CD19 and FITC anti-CD25 α chain.

2.11. PLASMA COLLECTION METHOD

The blood was removed as described in Section 2.3. The blood was transferred to eppendorfs and pulsed at 10000×g in a microfuge for 30 seconds. The top straw-coloured plasma layer was removed and stored at -70°C for further analyses.

2.12. VAT-SPECIFIC ANTIBODY LYTIC TITRES

Trypanosomes were extracted and purified as before (Section 2.3.) and were resuspended at 2×10^6 parasites/ml in PSG. Antibody-dependent complement-mediated lysis reactions were conducted essentially as described by McLintock *et al* (1993). Doubling dilutions of plasma and guinea pig serum (Seralab) as a source of complement were carried out in round-bottomed microtitre plates. 10µl of trypanosomes were added to each well and the plates were incubated for 1 hour at 37°C. Samples from each well were taken for examination by microscopy. The lytic titre is the reciprocal value of the highest antibody dilution which caused 50% lysis.

2.13. ANTIBODY ISOTYPING

Radioimmunodiffusion kits (The Binding Site, Birmingham) were used to quantify the amount of IgG_1, IgG_{2a}, IgG_{2b} and IgM present in the plasma of each mouse, according to the manufacturers instructions. 5µl/well of each sample was added, with the exception of the IgG_{2b} plate where 10µl/well was required, at the recommended dilutions. A set of

3 standards for each antibody isotype was used as supplied with the kits. The IgG_1 plates were incubated in a humid chamber at room temperature for 72 hours with the remaining plates incubated for a further 24 hours before the rings of precipitation were measured and the isotype amounts calculated.

2.14. ANTIBODY ABSORPTION STUDIES

Trypanosome VSG surface epitope-specific antibody isotype/subclass levels were determined as follows: Trypanosomes expressing defined VAT's were purified from rats over Percoll gradients as described in Section 2.3. These parasites were then used to absorb surface-epitope specific antibodies from plasma samples using a method based on that of Magnus *et al* (1982). The trypanosome layer was removed and washed twice in ice cold PSG by pulsing at 10000×g in a microfuge for 30 seconds. A pellet of live trypanosomes of approximately 100µl volume was mixed with 300µl of heat-inactivated (56°C for 30 minutes) plasma. The suspension was incubated on ice for 90 minutes, then microfuged as before. 15µl of plasma was removed and stored frozen for isotyping and the remainder of the plasma mixed with a second pellet which was then incubated for a further 90 minutes. After this incubation, the parasites were pelleted and the plasma removed and stored for isotyping as described in Section 2.13. Preliminary experiments showed that absorption was complete after 2 rounds.

2.15. GIEMSA STAINING

The smears from the peritoneal population were fixed in methanol for 5 minutes then air dried. They were submerged in PBS/H_20 at 50:50 (v/v), incubated at room temperature for 10 minutes and then placed directly into Giemsa staining solution of 20% Giemsa buffer (3g/L di-sodium hydrogen orthophosphate, BDH; 0.6g/L potassium di-hydrogen orthophosphate, BDH, and H_20), 70% water and 10% Giemsa stain (BDH). After a 15 minute incubation the slides were washed 3 times in tap water and allowed to air dry before examination by microscopy.

2.16. DRUG CURE OF TRYPANOSOME-INFECTED MICE

Cymelarsan was dissolved immediately prior to use in dH_20. A concentration of 0.5mg/ml was used and 0.4mls/mouse was i.p. injected giving a curative dose of 10mg/kg. The blood was monitored 24 hours later to ensure that all the trypanosomes had been killed.

2.17. PARASITAEMIA DETERMINATION

During acute monomorphic infections, the parasitaemias were monitored daily by the 'rapid matching' method of Herbert & Lumsden (1976). To determine parasitaemias accurately in chronic infections, mice were monitored by venesection of the tip of the tail and removal of 2µl of blood daily. The blood was diluted to an appropriate concentration in 0.1M ammonium chloride and the trypanosomes counted with an Improved Neubauer haemocytometer.

2.18. PREPARATION OF TRYPANOSOME LYSATES

The trypanosomes were extracted and purified as previously described (Section 2.3.). The trypanosomes were counted and resuspended at 10^8/ml in PBS pH 7.4. 1ml aliquots were made which were subjected to 3 cycles of freeze-thawing in liquid nitrogen and water at 60°C. The lysates were microfuged for 30 seconds at 10000×g to remove insoluble material. The trypanosome lysates were then used in the proliferation assays.

2.19. SELECTION OF POTENTIAL T-CELL EPITOPES IN VSG MOLECULES

The amino acid sequences of five *T.brucei* VSGs, ILTat 1.22, ILTat 1.24, ILTat 1.3, ILTat 1.61 and MITat 1.2 (Molteno Institute *Trypanozoon* antigen type 1.2) (Rice-Ficht *et al*,1981; Carrington *et al*,1991; Carrington, personal communication) were analysed.

The sequences for ILTat 1.24 and MITat 1.2 were used because the tertiary structure for these is already known and ILTat 1.22, ILTat 1.3 and ILTat 1.61 were used because they are readily available in our laboratory. The potential epitopes were selected on the primary amino acid sequences using a computer program, "TSites" (Feller & La Cruz,1991), and mapped onto ribbon drawings of the tertiary structures of MITat 1.2 (Freymann *et al*,1990) and ILTat 1.24 (Blum *et al*,1993). This programme compares the predicted T-cell epitopes on proteins using four separate algorithms.

The "AMPHI" motif, "A", identifies amphipathic helices (Margalit *et al*,1987) within the VSG and this search is based on the theory that T-cell epitopes are predominantly amphipathic helices (Delisi & Berzofsky,1995).

The "R" motif identifies two peptide configurations of four and five amino acids each. The first sequence consists of a charged/glycine-hydrophobic-hydrophobic-polar/glycine motif and the second consists of a charged/glycine-hydrophobic-hydrophobic-hydrophobic/proline-polar/glycine motif (Rothbard & Taylor,1988). Both these algorithms identify helper and cytotoxic T-cell epitopes.

The I-A^d and I-E^d motifs, "D" and "d" respectively, predict peptide sequences likely to bind to major histocompatibility complex (MHC) Class II molecules. These motifs can be recognised independently on the primary protein sequence (Sette *et al*,1989).

A computer program, "Rasmol", generated the tertiary VSG structure in ribbon form and the potential T-cell epitopes were mapped onto this. These potential peptides could therefore be identified to specific regions of the VSG tertiary structure.

2.20. CONJUGATION OF PEPTIDES TO KEYHOLE LIMPET HAEMOCYANIN CARRIER

Four individual peptides predicted to correspond to T-cell epitopes on VSG molecules were synthesised (Genosys Biotechnologies Ltd, Cambridge, UK) each consisting of 14 amino acids followed by a cysteine residue for C-terminal amidation. The peptides were added in excess to the Keyhole Limpet Haemocyanin (KLH) (Pierce). To do this, 2mgs of each peptide was disolved in 500µl of a solution of PBS pH 8.0

containing a minimum concentration (no more than 30%) of Dimethyl Sulfoxide, v/v, (Sigma) required to solubilise the peptide. Vials containing 2mgs of KLH were reconstituted in 200µl of dH_20 thus activating the KLH. Each peptide solution was then immediately combined with an individual vial of KLH and mixed for 2 hours on a shaker at room temperature.

To remove any EDTA from this activated KLH, the conjugates were passed through D-Salt Extracellulose Plastic Desalting columns (Pierce). For each peptide, a column was equilibrated with 5 column volumes of PBS pH 8.0, conjugate added, columns eluted with PBS pH 8.0, 1ml aliquots collected and their absorbance read at a wavelength of 280nm by spectrophotometry. Aliquots containing protein were pooled and used for immunisation of mice.

2.21. IMMUNISATION PROCEDURE

The peptide conjugates in PBS pH 8.0 were mixed at a 1:1 ratio with Complete Freund's adjuvant (Sigma) to allow initial immunisations of 40µg/mouse in 0.2ml volumes. The solutions were mixed using an Ultra-Turrax Drive microprobe (Janke & Kunkel, IKA-Laborkchnik) at a speed of 24000 rpm to combine completely the adjuvant with the conjugated peptides. The same procedure was used for secondary immunisations but using Incomplete Freund's adjuvant 2 weeks later. The mice were killed 2 weeks after secondary immunisation.

Table 2.1a. Primary and secondary positive control murine antibodies used for immunofluorescence antibody technique.

	PRIMARY ANTIBODY		SECONDARY ANTIBODY	
VAT	POSITIVE CONTROL	DILUTION FACTOR	FITC ANTIBODY	DILUTION FACTOR
ILTat 1.3	Monoclonal Ab 23.2	Neat	Anti-mouse	1/50
ILTat 1.61	Monoclonal Ab 23.1	Neat	Anti-mouse	1/50
GUTat 7.1	Monoclonal Ab 27.1	Neat	Anti-mouse	1/50
GUTat 7.2	Monoclonal Ab 18.7	Neat	Anti-mouse	1/50

Table 2.1b. Negative control primary and secondary murine antibodies for immunofluorescence.

	PRIMARY ANTIBODY		SECONDARY ANTIBODY	
VAT	NEGATIVE CONTROL	DILUTION FACTOR	FITC ANTIBODY	DILUTION FACTOR
ILTat 1.3	Monoclonal Ab 18.7	Neat	Anti-mouse	1/50
ILTat 1.61	Monoclonal Ab 27.1	Neat	Anti-mouse	1/50
GUTat 7.1	Monoclonal Ab 23.1	Neat	Anti-mouse	1/50
GUTat 7.2	Monoclonal Ab 23.1	Neat	Anti-mouse	1/50

All monoclonal antibodies were used as hybridoma culture supernatants.

Table 2.1c. Rabbit antibodies used for immunofluorescence antibody technique as the positive control.

	PRIMARY ANTIBODY		SECONDARY ANTIBODY	
VAT	POSITIVE CONTROL	DILUTION FACTOR	FITC ANTIBODY	DILUTION FACTOR
ILTat 1.3	Rabbit Anti-ILTat 1.3	1/200	Anti-rabbit	1/100
ILTat 1.61	Rabbit Anti-ILTat 1.61	1/200	Anti-rabbit	1/100
GUTat 7.1	Rabbit Anti-GUTat 7.1	1/200	Anti-rabbit	1/100
GUTat 7.2	Rabbit Anti-GUTat 7.2	1/200	Anti-rabbit	1/100
ANTat 1.8	Rabbit Anti-ANTat 1.8	1/200	Anti-rabbit	1/100

Table 2.1d. Primary and secondary rabbit IFAT antibodies used as negative controls.

	PRIMARY ANTIBODY		SECONDARY ANTIBODY	
VAT	NEGATIVE CONTROL	DILUTION FACTOR	FITC ANTIBODY	DILUTION FACTOR
ILTat 1.3	Rabbit Anti-GUTat 7.1	1/200	Anti-rabbit	1/100
ILTat 1.61	Rabbit Anti-GUTat 7.2	1/200	Anti-rabbit	1/100
GUTat 7.1	Rabbit Anti-ILTat 1.3	1/200	Anti-rabbit	1/100
GUTat 7.2	Rabbit Anti-ANTat 1.8	1/200	Anti-rabbit	1/100
ANTat 1.8	Rabbit Anti-ILTat 1.61	1/200	Anti-rabbit	1/100

Figure 2.1. The pedigree diagrams describing the life histories of the trypanosome stocks for monomorphic lines ILTat 1.3 (a), ILTat 1.61 (b), GUTat 7.1 (c), GUTat 7.2 (d) and pleomorphic line GUTat 7.2 (e) used throughout this study are shown. They are drawn according to the conventions of Lumsden *et al* (1973). Cryopreserved stabilate numbers appear in boxes, with primary isolates being designated by the source, date and place of isolation. Solid lines represent passage in animals, where (M) represents mice, and broken lines indicate cloning of trypanosomes. Double lined arrows represent cyclical transmission by tsetse flies. Stabilates containing VAT reference populations are designated by cartouches. Neutralisation reactions *in vitro* are also shown (N) and the antiserum preparation used are stated.

Figure 2.1a. Derivation of cloned monomorphic line ILTat 1.3.

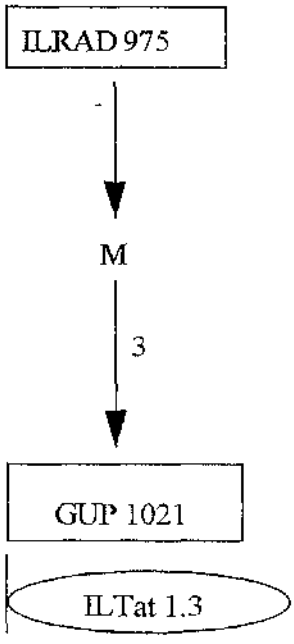


Figure 2.1b. Derivation of cloned monomorphic line ILTat 1.61.

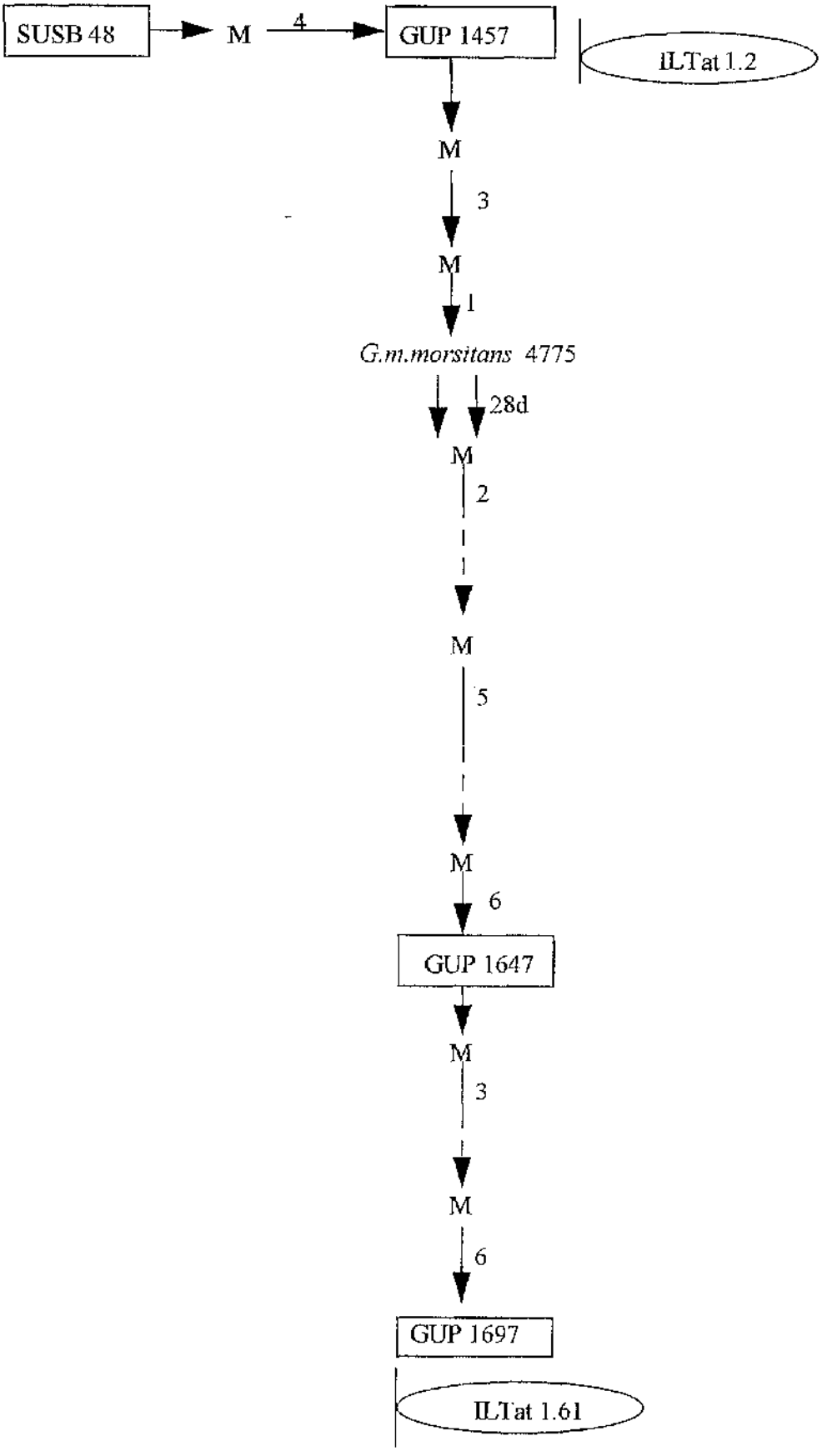
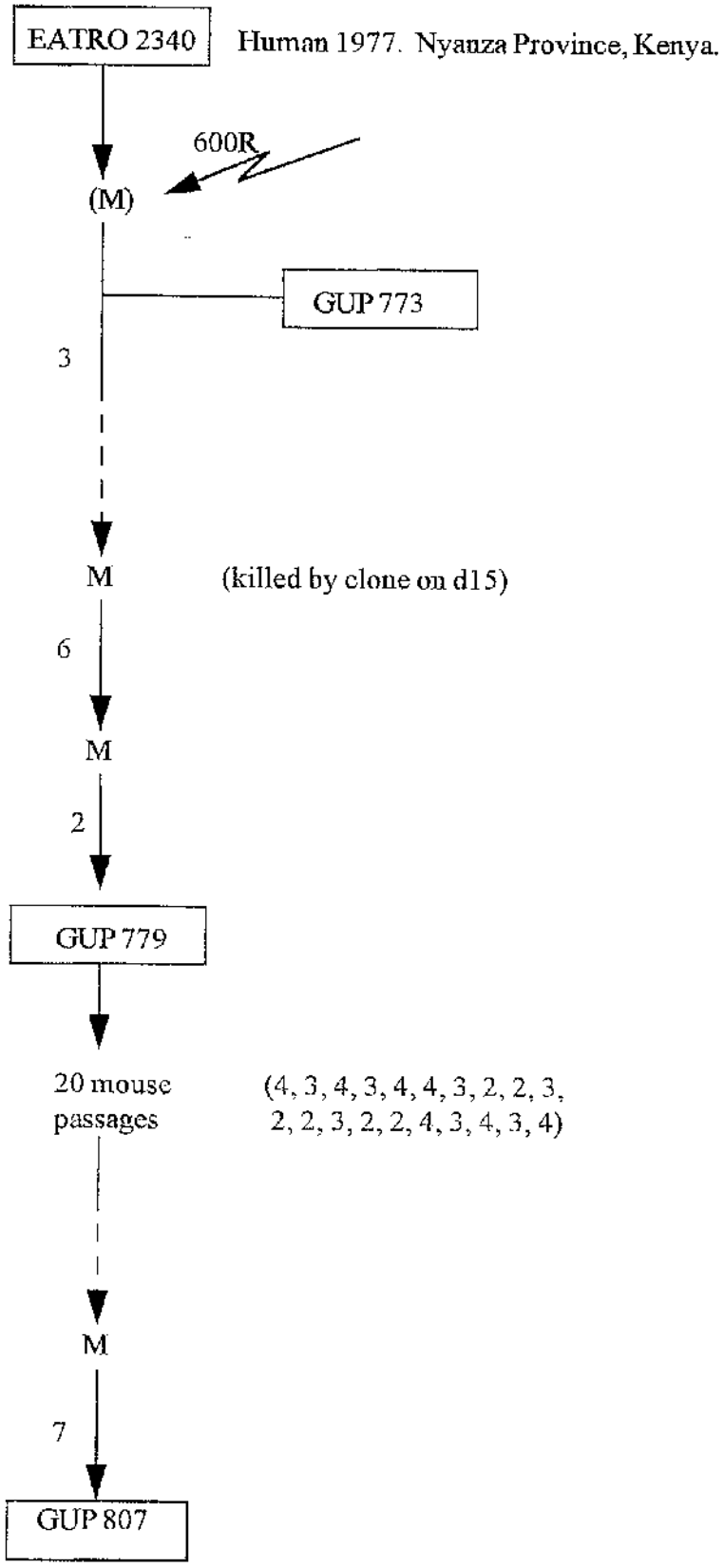


Figure 2.1c. Derivation of cloned monomorphic line GUTat 7.1.



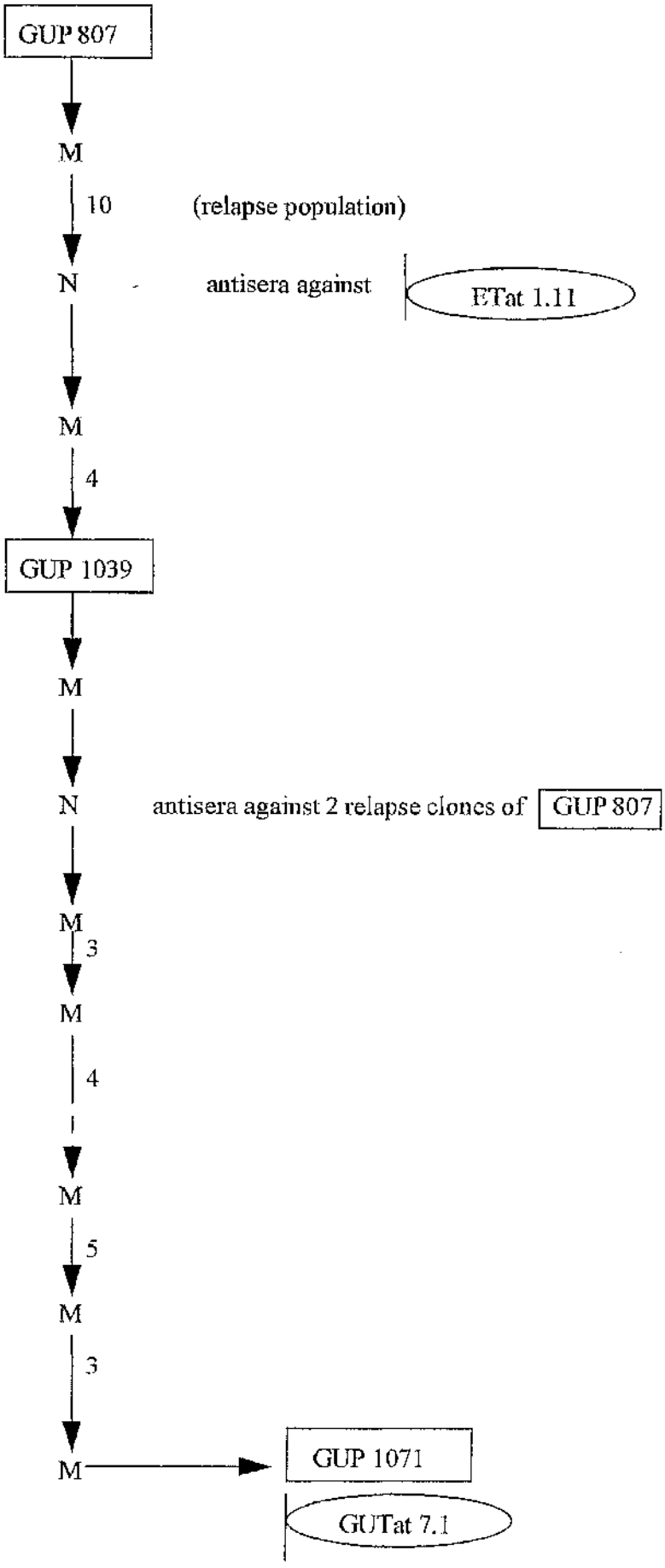

GUP 807
M
10
(relapse population)
N
antisera against
ETat 1.11
M
4
GUP 1039
M
N
antisera against 2 relapse clones of
GUP 807
M
3
M
4
M
5
M
3
M
GUP 1071
GUTat 7.1

Figure 2.1d. Derivation of cloned monomorphic line GUTat 7.2.

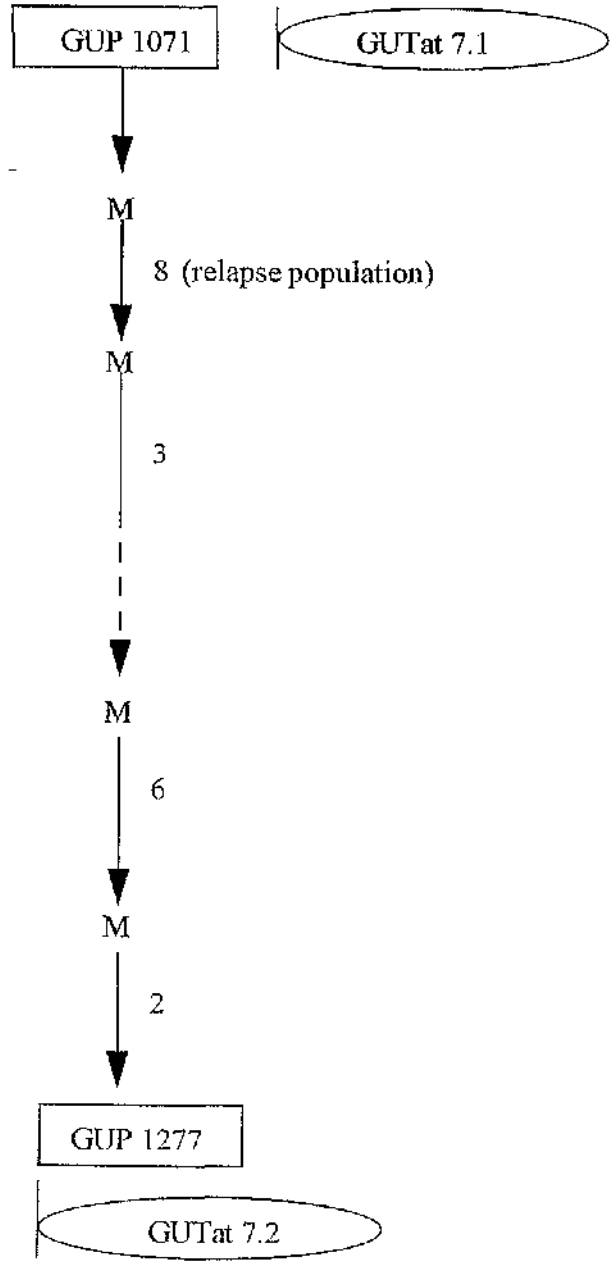
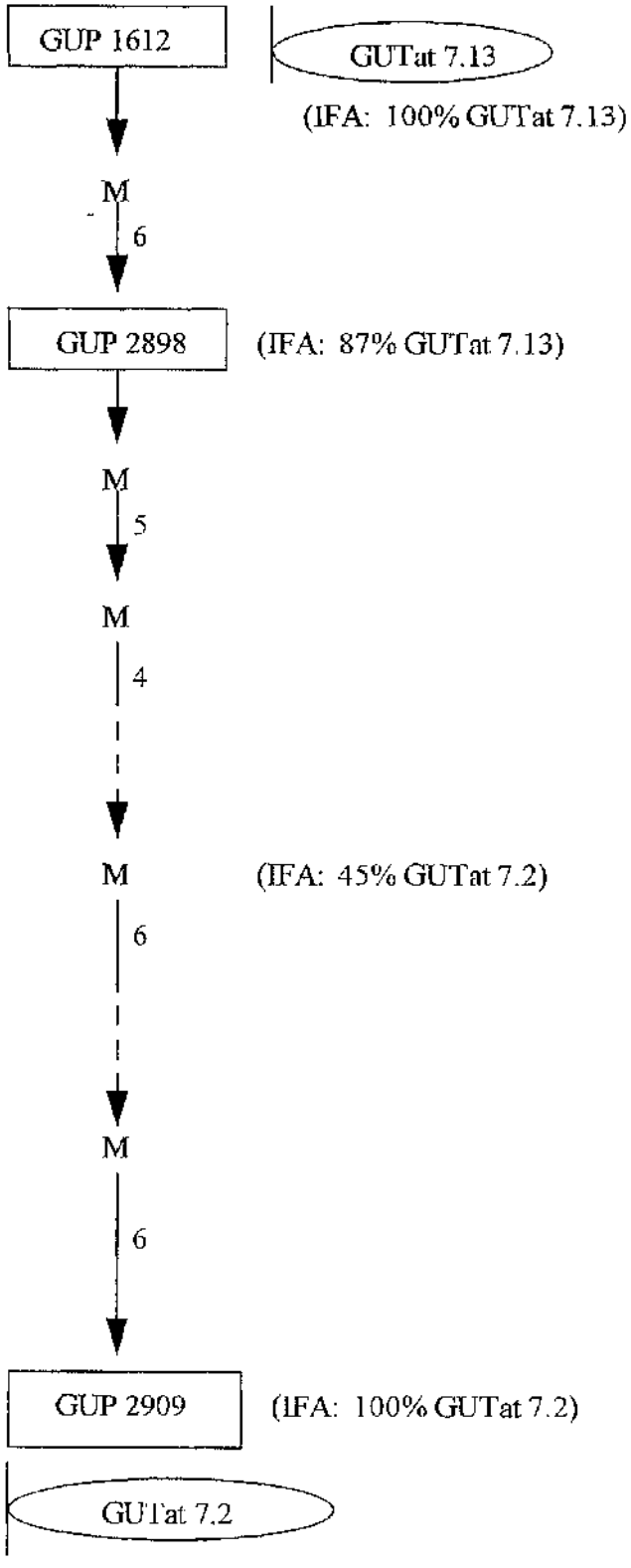


Figure 2.1e. Derivation of cloned pleomorphic line GUTat 7.2.



CHAPTER 3

DEVELOPMENT OF AN *IN VITRO* MODEL SYSTEM FOR INVESTIGATING PROLIFERATIVE T-CELL RESPONSES TO TRYPANOSOME INFECTIONS

3.1. INTRODUCTION

There has been no efficient way to examine antigen-specific T-cell proliferative responses to African trypanosomes in an *in vitro* environment as yet. A number of different approaches have been described but the extent to which most of these bear comparison with the *in vivo* situation is debatable.

Several studies have opted for the straightforward option of investigating the T-cell proliferative responses using cells from various murine infected lymphoid organs by stimulating with the mitogens Con A or Pokeweed Mitogen (PWM) and comparing these responses with those of uninfected mice (Gasbarre *et al*,1980; Kar *et al*,1981; Sileghem *et al*,1986; Borowy *et al*,1990; Flynn & Sileghem,1991; Sternberg & McGuigan,1992; Mabbott *et al*,1995).

A few investigations have examined responses to non-trypanosome antigens. Sileghem *et al* (1986) looked at T-cell proliferation in response to antigen- and Con A-induced IL-2 production comparing uninfected and trypanosome-infected mice. The only antigenic stimulus used in these experiments was Ovalbumin, rather than trypanosome antigens and therefore the specificity of response for trypanosomes remained unexplored. Sztein & Kierszenbaum (1991) examined the response of

peripheral blood mononuclear cells (PBMCs) when they were incubated in the presence of Phytoheamaggluttanin (PHA) and supernatants, from *T.b.rhodesiense* cultures, compared with PBMCs which were incubated with PHA alone. These experiments were used to see if the supernatants contained a secreted product which prevented T-cell proliferation by inducing immunosuppression. Other groups have mixed the lymphoid cells of trypanosome-infected mice with sheep RBCs and measured the T-cell proliferative response (Clayton *et al*,1979; Wellhausen & Mansfield,1980).

Live trypanosomes have also been used in *in vitro* assay systems to examine the proliferation of T-cells in response to trypanosomes (Gasbarre *et al*,1980; Kierszenbaum *et al*,1991; Sileghem *et al*,1991). Gasbarre *et al* (1980) claimed that live trypanosomes were necessary for priming *in vivo* and for activation *in vitro* and therefore stimulated their T-cells *in vitro* with live trypanosomes. However, Clayton *et al* (1979) used irradiated trypanosomes, which are non-replicating, to mimic the effect of the live parasites to successfully stimulate T-cell proliferation. This system seems to be the nearest to an *in vivo* situation with the exception of using live trypanosomes. Lymphocyte stimulation, rather than T-cell proliferation, has been examined with respect to the induction of murine macrophages procoagulant activity during *T.brucei* infections (Rossi *et al*,1987). Medium alone, Con A or live trypanosomes were used as the *in vitro* stimuli.

Many other groups have used trypanosome fractions but the rationale for choice of fractions made is sometimes less than obvious in that it is difficult to envisage what the T-cell responses, if any, will correlate to *in situ*. Olsson *et al* (1993) used a *T.b.brucei*-released 'lymphocyte triggering factor' as an antigenic stimulus in comparison with medium and Con A. They appear not to have determined however whether this triggering factor is present and the same in all trypanosome infections. They also did not

use a parasite surface antigen as a comparison in their proliferation assays. One reason for this may be that it has proved surprisingly difficult to induce proliferation of T-cell responses using trypanosome VSG despite the fact that it is surface located in the parasites and the most abundant protein. Soluble monomeric VSG purified from viable bloodstream trypanosomes, at concentrations varying from 0.1μg/ml to 100μg/ml of VSG , has been used as antigenic stimulus by Schleifer *et al* (1993). They also used a trypanosome lysate as an additional stimulus and compared these to Con A-induced T-cell proliferation. The mice used had been infected for two weeks before being used in the proliferation assays and stimulated with either Con A, monomeric VSG or the lysate and their results showed that there was no proliferation. The T-cell responses of trypanotolerant and trypanosusceptible cattle infected with *T.congolense* have been examined (Flynn *et al*,1992). As an antigenic stimulus, whole trypanosome lysates were fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the major bands were isolated and used directly in the proliferation assays. Flynn *et al* (1992) showed that their VSG fraction did not induce T-cell proliferation despite the presence of serum antibodies to the VAT used. The VSG band induced very little or no proliferation in both the trypanotolerant or trypanosusceptible cattle.

There is therefore a necessity to develop an *in vitro* proliferation assay system the results of which would be more readily extrapolated to *in vivo* situations and yet avoids the need to use live trypanosomes. An ideal system would allow analyses of the T-cell proliferative responses against the areas of the trypanosome which are directly seen by the host, that is the VSG surface coat which forms a physical barrier between the parasite plasma membrane and the host thus protecting the parasite from non-specific immune attack. In this way, it would be possible to investigate the apparent paradox of an abundant, surface located protein failing to induce proliferation of T-cells.

The assay should be as close as possible to *in vivo* situations which therefore rules out the use of lysates and VSG fractions because most antigenic challenge in an infection can reasonably be assumed to come from live parasites rather than solubilised remains of dead ones. Intact parasites would be best but for pragmatic reasons (the need to avoid growing trypanosomes in mice to the correct parasitaemia, purify them from blood and check VAT expression in the same day as preparing mononuclear cells for proliferation analyses) I was keen to avoid using live parasites routinely. Also, the optimal media for growth of trypanosomes and splenocytes are very different in that trypanosome media are primarily Minimum Essential Medium based (Baltz *et al*,1985), with a range of additional supplements, whereas splenocyte culture media are usually RPMI based. I therefore attempted to develop a system that allows trypanosome VAT-specific responses to be compared in an easy, efficient and inexpensive manner using intact parasites which are non-infective and non-replicating , that is they are dead but still intact and with the integrity of the antigenic epitopes still preserved as far as possible. The T-cell proliferative response to each trypanosome VAT could then be compared with each other and with response to a mitogenic stimulus.

Because African trypanosomes are extracellular parasites in the bloodstream, the spleen is the most obvious lymphoid organ to examine. A decision was made to investigate proliferative responses in mononuclear splenocyte preparations rather than to purify T-cells to a homogeneous population. This type of proliferation assay relates to T-cells only and is readily associated with accessory cells (Askonas & Bancroft,1984). This approach has been used widely (and successfully) in a variety of immunological models: *Mycobacterium tuberculosis* (Vordermeier *et al*,1993); *Leishmania major* (Wei *et al*,1996); *Brugia pahangi* (Osborne *et al*,1996); *Cryptosporidium muris* (Davami *et al*,1997); *Trypanosoma brucei* (Black *et al*,1989; Bakhiet *et al*,1993b).

3.2. RESULTS

A number of variables that could reasonably be expected to affect the performance of an antigen-driven proliferative assay were investigated.

3.2.1. Incubation Time

The optimal incubation time before harvesting for the proliferation assays was found to be 72 hours with the cells being pulsed 16 hours prior to harvesting [Figure 3.1.]. This timepoint was chosen as the negative control, medium, showed very little proliferation whereas Con A, the positive control, showed high levels of proliferation. The other time points were disregarded because after 24 hours the medium control counts were above 20,000 cpm, a probable result of the trauma of being removed and purifed, and although this factor wained, at 48 hours it still remained at 5,000 cpm. 72 hours was the minimum time that gave a reliable negative control and produced a high response to Con A.

3.2.2. Concanavalin A Concentration

The optimal Con A concentration was 8mg/ml when used with the optimal incubation time, 72 hours [Figure 3.2.]. Concentrations of Con A lower than 8µg/ml generated

very little proliferative response of the mononuclear splenocyte populations and levels of response using 12-16μg/ml were similar to those observed using 8μg/ml.

3.2.3. Trypanosome Antigenic Stimuli

Mononuclear splenocytes harvested from GUTat 7.2-infected mice were stimulated with medium alone and with three different preparations of trypanosome antigen [Figure 3.3a.]. The stimulation with medium resulted in background levels of proliferation whereas the live and paraformaldehyde-fixed trypanosomes produced a proliferative response several fold higher than that of the medium control. The trypanosome lysate created the greatest degree of proliferation of the three preparations.

Using a trypanosome lysate in the T-cell proliferation assay resulted in immense levels of proliferation [Figure 3.3a.], presumably due to the simultaneous assault on the splenocytes by a large number of trypanosome antigens. Paraformaldehyde-fixed trypanosomes could stimulated the splenocytes to proliferate to a degree similar to that of the other preparations and were therefore used as the antigenic stimuli during all subsequent experiments. These paraformaldehyde-fixed trypanosome preparations can be stored in PBS pH 7.4 at 4°C for up to four weeks thus making it possible to prepare the antigenic stimuli before the day of experiment.

The optimal concentration of antigen for proliferation assays using paraformaldehyde-fixed GUTat 7.2 (monomorphic) trypanosomes was determined. As Figure 3.3b. shows, a concentration of fixed trypanosomes at 2×10^6/ml resulted in the highest proliferation of these splenocytes with concentrations 4×10^6/ml and 10^6/ml producing several fold lower levels of proliferation.

The epitopes on the VSG appear to remain intact during the paraformaldehyde fixation process. The evidence for this comes from immunofluorescence comparison of live, ethanol-fixed and paraformaldehyde-fixed trypanosomes [Table 3.1.]. ILTat 1.3 and GUTat 7.1 trypanosomes were examined using rabbit antibodies for each, as a positive control, and mouse monoclonal antibodies which identify subsurface epitopes for each antigen type. VSG-specific subsurface epitopes were available for only these two VATs. The ILTat 1.3 rabbit antibodies recognised the ILTat 1.3 trypanosomes in each of the three states but did not recognise GUTat 7.1 trypanosomes and vice versa for the rabbit antibodies to GUTat 7.1. The ILTat 1.3 monoclonal anti-23.2 antibody recognised regions on the ILTat 1.3 ethanol-fixed smears but not on the live or paraformaldehyde-fixed trypanosomes and did not recognise any regions on any forms of the GUTat 7.1 trypanosomes. The same was true for the GUTat 7.1 monoclonal anti-17.2 antibody which recognised the GUTat 7.1 ethanol-fixed trypanosomes but not the other forms and did not recognised any of the ILTat 1.3 forms. These data suggest that the epitope structure and organisation on the trypanosome VSG have been maintained by the paraformaldehyde fixation process.

Taken together, these data suggest that paraformaldehyde-fixed trypanosomes can generate proliferative responses and should be capable of doing so in a VAT-specific manner. They do not rule out the formal possibility however, that paraformaldehyde fixation modifies *any* cell such that it will drive proliferation. To investigate this possibility, I examined the proliferative responses driven by paraformaldehyde-fixed cells that were antigenically distinct from trypanosome - sheep RBCs.

3.2.4. Paraformaldehyde-Induced Proliferation By Sheep RBCs

The unfixed sheep RBCs induced the same amount of T-cell proliferation as the paraformaldehyde-fixed RBCs [Figure 3.4.]. When a stimulation index was used to examine these figures, Con A produced an index of 4.9 whereas the sheep RBCs produced an index of 1.0 and the fixed RBCs resulted in an index of 0.9. These data therefore suggest that the fixation and washing processes involved in preparing these cells, and by inference the trypanosomes, as antigenic stimuli for the proliferation assays do not cause the cells to proliferate non-specifically.

In summary, optimal incubation time and concentration of Con A (for use as a positive control) have been determined and I have estabilished that paraformaldehyde-fixed trypanosomes were capable of generating a proliferative response which was attributable to the antigen itself rather than the antigen fixation process. These preliminary data have provided a basis for investigating whether trypanosome-specific proliferative reponses could be detected in splenocytes from infected mice and the degree to which they may be VAT-specific.

3.2.5. T-cell Proliferation Assay System Using Mononuclear Splenocytes From Uninfected And Infected Mice

The lymphocytes in the mononuclear splenocyte suspensions from ILTat 1.3-infected mice proliferated to a different degree with each of the two antigen types used as an *in vitro* stimuli. Using the splenocytes from the uninfected mice, there was minimal proliferation when the cells were stimulated with medium alone and high levels of proliferation when stimulated with Con A, >14,000 cpm. There were differing, but low

level, degrees of proliferative responses when stimulated with the trypanosome VATs [Figure 3.5a.]. When the cells were stimulated *in vitro* with ILTat 1.3 there was a response of 7,000 cpm whereas with ILTat 1.61 the response was 5,000 cpm.

When the mononuclear splenocytes were purified from mice infected with ILTat 1.3 trypanosomes at the peak of parasitaemia and stimulated *in vitro* with medium alone, Con A, ILTat 1.3 or ILTat 1.61 fixed trypanosomes different responses were observed when compared with those of the uninfected mice [Figure 3.5b.]. There appeared to be far greater proliferation with the cells stimulated with medium alone than with lymphocytes from uninfected mice. This I attribute to the fact that the cells were highly primed, due to the high parasite yield in the host, and the probable carry-over of processed antigen into the *in vitro* environment. The responses to Con A were also highly elevated rising from <20,000 cpm in uninfected mice to >200,000 cpm in infected mice. There was a dominant homologous antigen-driven response to the ILTat 1.3 paraformaldehyde-fixed trypanosomes and a lack of a T-cell proliferative response to the heterologous ILTat 1.61 trypanosomes.

These data suggest that trypanosome VAT-specific proliferative responses are detectable in mononuclear splenocytes from trypanosome-infected mice using the assay system developed. The use of this assay system to investigate variant antigen specificity and mechanisms of T-cell responses will be addressed in Chapters 4, 6 and 7.

3.3. DISCUSSION

Although a number of groups have examined T-cell proliferative responses during *T.brucei* infections, surprisingly little work has been conducted on antigen-driven

proliferative responses. This is possibly because there is a lack of a reliable *in vitro* assay system which can compare mitogen-driven responses as well as trypanosome antigen-driven responses. The *in vitro* system described here can be used reliably to investigate T-cell responses which are VAT-specific during African trypanosome infections. It does not rely on comparing mitogen responses of infected and uninfected mice but uses intact VSG which is still expressed on the trypanosome surface. Immunofluorescence microscopy analysis has suggested that the VSG coat on the trypanosomes appears to maintain elements of its native integrity. The host cells should therefore be able to process the antigenic VSG into peptides and present those which fit into the MHC groove to induce an immune response in a manner similar to that which would occur in an infection.

Using sheep RBCs to examine the T-cell response during African trypanosomisis seems completely irrelevant as it explains nothing about the T-cell response to trypanosomes or to trypanosome antigens (Clayton *et al*, 1979; Wellhausen & Mansfield, 1980). It will only serve as indicator of depression or increased responsiveness of the T-cells from mice which have been subjected to a trypanosome infection.

Live trypanosomes would provide a closer comparison to *in vivo* situations than paraformaldehyde-fixed cells but the live trypanosomes might potentially induce suppression of the cells thereby inhibiting the immune responses. Also, if several VATs are to be compared in an experiment then it becomes more difficult to obtain all the required trypanosome lines at the same time due to the differences in growth rates. The optimal cell culture media for splenocytes would provide an inadequate environment for live trypanosomes and this would inevitably result in the trypanosomes lysing and

releasing large amounts of internal antigens which would not normally be seen. This would obviously effect the proliferation and cytokine results.

Irradiated trypanosomes could be of use if the VSG surface coat is left intact after the irradiation process. It would have to be known how long these irradiated trypanosomes could be kept for or if they have to be used immediately after they are irradiated. It is also a more difficult and more time consuming process compared to mild chemical fixation.

The assay system described here has been shown to produce proliferative T-cell responses which are antigen-driven by using mononuclear splenocytes and stimulating *in vitro* with medium alone, mitogen, or two different VATs, each expressing different antigen types. These responses were detected by using mononuclear splenocytes from trypanosome-infected mice at the first peak of parasitaemia, before immunosuppression begins and before more than one antigen type is present as the homotypic population in the bloodstream.

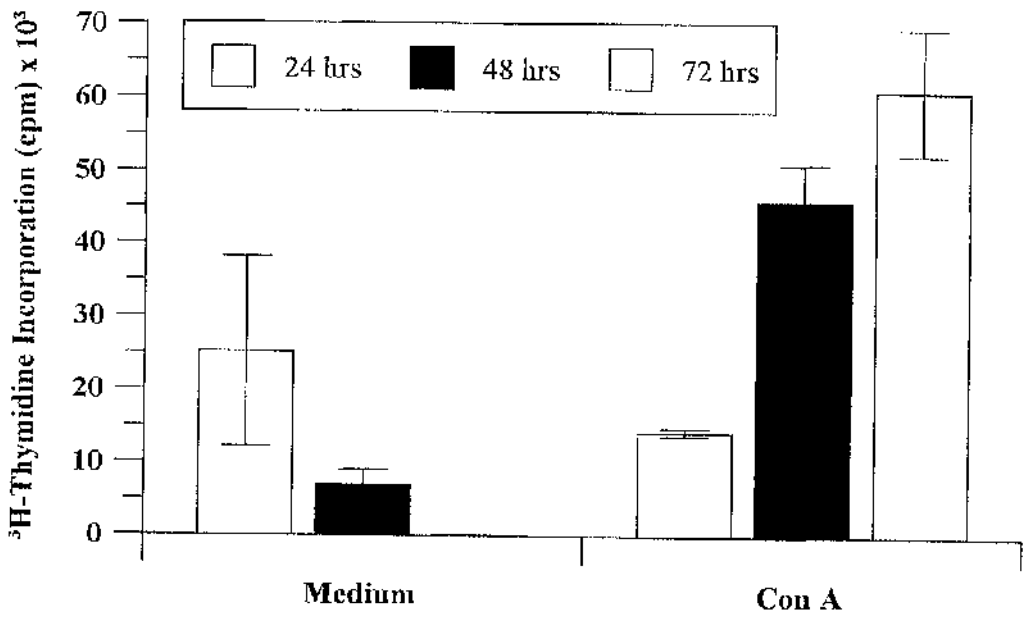
It is not surprising that Schleifer *et al* (1993) found no T-cell proliferation in their experiments when they stimulated with purified VSG or trypanosome lysates as the murine cells will have been immunosuppressed *in vivo* due to the mice being infected for two weeks before analysis and would not have had time to recover from the suppression in *in vitro* culture. It would therefore be expected that little or no T-cell proliferation, no matter what the antigenic stimuli, would be produced under these conditions because of the remarkable degree of suppression that a trypanosome infection can cause (Corsini *et al*,1977; Jayawardena *et al*,1978; Grosskinsky *et al*,1983; Silcghem *et al*,1986; Schleifer & Mansfield,1993).

A trypanosome lysate would in any case represent an unrealistic method of antigen presentation because of the large amounts of internal antigens present in the parasites

and their sudden disclosure to the host cells. These internal antigens are not usually seen by the immune system until phagocytosed by macrophages or internalised by antigen presenting cells (APCs) for presentation to T-cells and this potential difference in method of presentation could thus influence the response. Also, the earliest reported detection of internal trypanosome components was at day seven post-infection but was routinely several days after this, usually day 10 (Pearson *et al*,1986). The first peak of parastiamaemia in the trypanosome lines used in Chapters 4 and 6 are always before day seven so a lysate would be less realistic than fixed parasites. It would be very difficult to discriminate between the proliferation which is caused by the internal antigens and that which is VAT-specific due to epitopes on the VSG. Macrophages will be the principal APCs involved with processing and presenting trypanosomes during infection as they actively phagocytose large particles like parasites and bacteria whereas specific B-cells will present antigen *in vivo* if the antigen is at a low concentration (Abbas *et al*, 1991) but low antigen concentration is not the case during *T.brucei* infections. Macrophages play an active role in immunosuppression during trypanosome infections whereas there is no evidence for such a role by B-cells therefore a pathway involving macrophages is the prefered choice (Sileghem *et al*,1991; Sternberg & McGuigan,1992; Darji *et al*,1993; Schleifer & Mansfield,1993; Mabbott *et al*,1995).

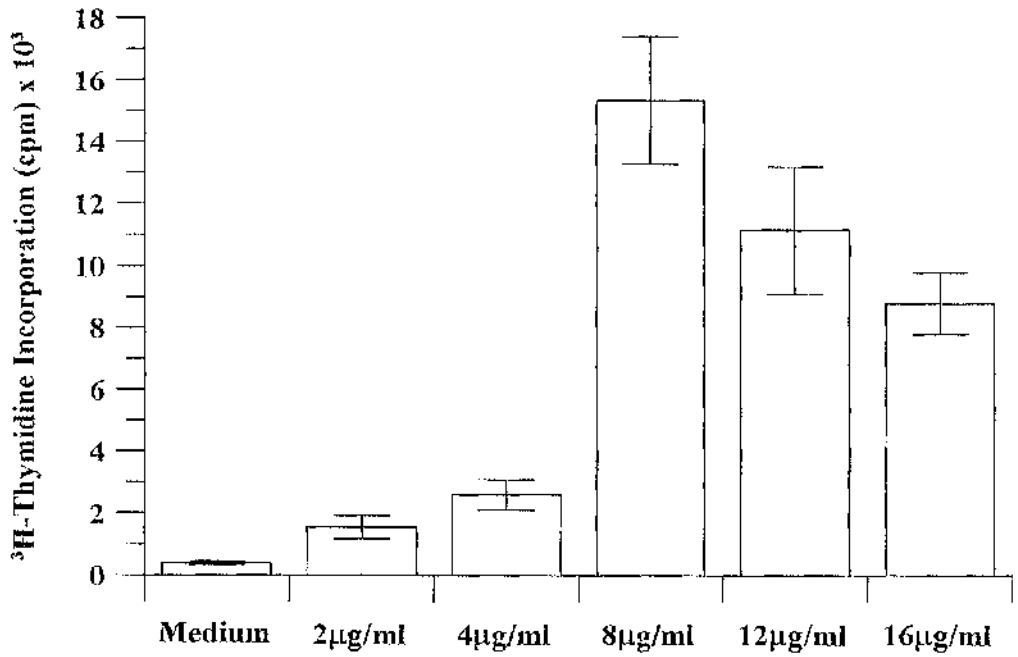
To the best of my knowledge there has been no better *in vitro* proliferation system developed for examining T-cell proliferative responses to African trypanosomes. Immunodepression can however be investigated by comparing the Con A responses but this approach is severely constrained in generating understanding of protective immune responses of the host and VAT-specific T-cell proliferation. It allows the response to an individual trypanosome VAT to be analysed rather than using only an irrelevant antigen like the sheep RBCs or a complex of antigens as present in lysates.

Figure 3.1. Optimum *in vitro* incubation time of mononuclear splenocytes for a proliferation assay.



Proliferation of mononuclear splenocytes from GUTat 7.2-infected mice 3 days post-infection. The lymphocytes were dispensed at 5×10^5 cells/well and were stimulated with culture medium alone or Con A (8μg/ml) for either 24, 48 or 72 hours. ^{3}H-Thymidine incorporation determined T-cell proliferation in each case. Results are expressed as the means of quadruplicate wells ± 2 SE.

Figure 3.2. Optimum concentration of Concanavalin A for proliferation of mononuclear splenocytes.



The concentration of Con A to be used continuously in this proliferation assay system was determined by incubating mononuclear splenocytes, 5×10^5 cells/well, from uninfected mice for 72 hours, stimulating with culture medium alone and varing concentrations, 2-16µg/ml, of Con A. The results are expressed as the means of quadruplicate wells ± 2 SE.

Figure 3.3. Mononuclear splenocytes were harvested from GUTat 7.2-infected mice and dispensed at 5×10^5 cells/well. To determine the best form of preparation in which the trypanosome antigens would be used, the splenocytes were stimulated with live homologous trypanosomes (2×10^6/ml), a trypanosome lysate of GUTat 7.2 trypanosomes (made from 2×10^6/ml) and 2×10^6/ml paraformaldehyde-fixed GUTat 7.2 trypanosomes (**a**). When the decision to use paraformaldehyde-fixed parasites was made, the optimal numbers of trypanosomes to use was determined by stimulating the mononuclear splenocytes with three different concentrations of paraformadehyde-fixed parasites (**b**). The results are expressed as the means of quadruplicate wells ± 2 SE.

Figure 3.3a. Comparison of trypanosome antigen preparations for stimulating proliferation of mononuclear splenocytes.

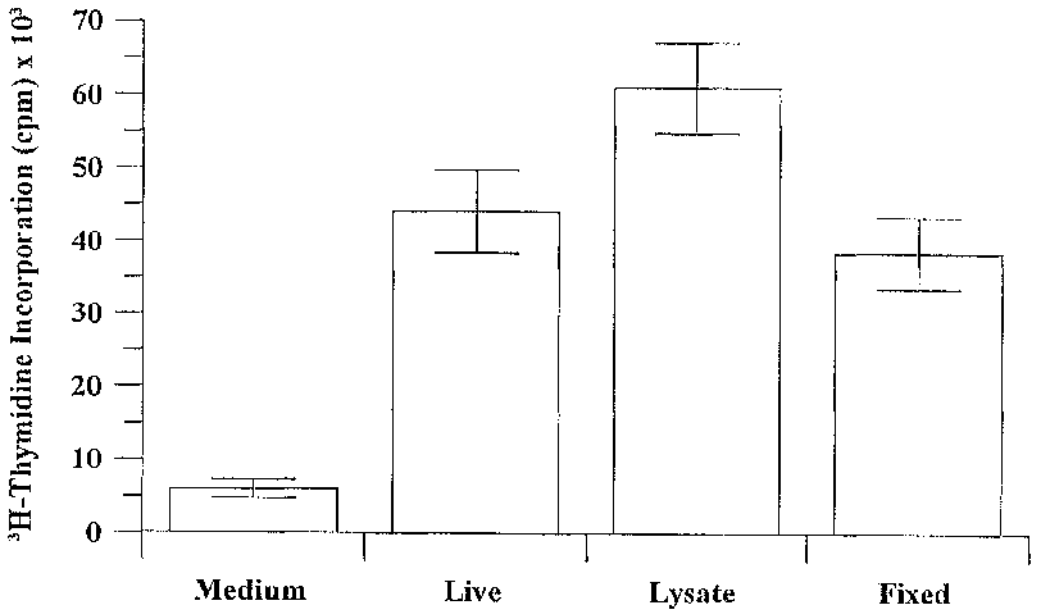


Figure 3.3b. Number of paraformaldehyde-fixed trypanosomes required to induced optimal proliferation.

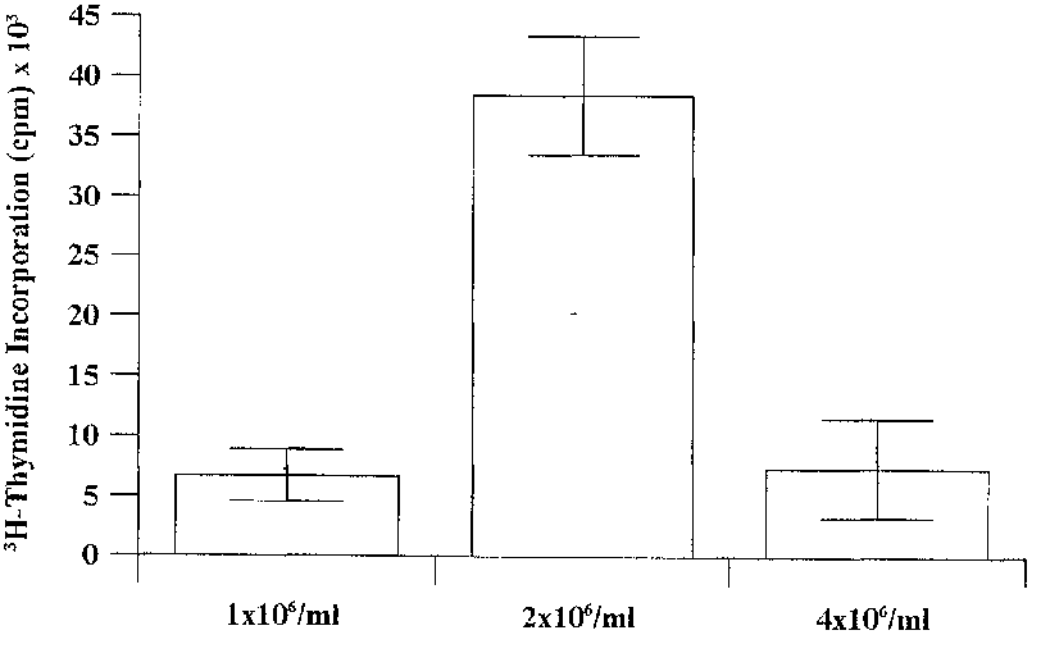
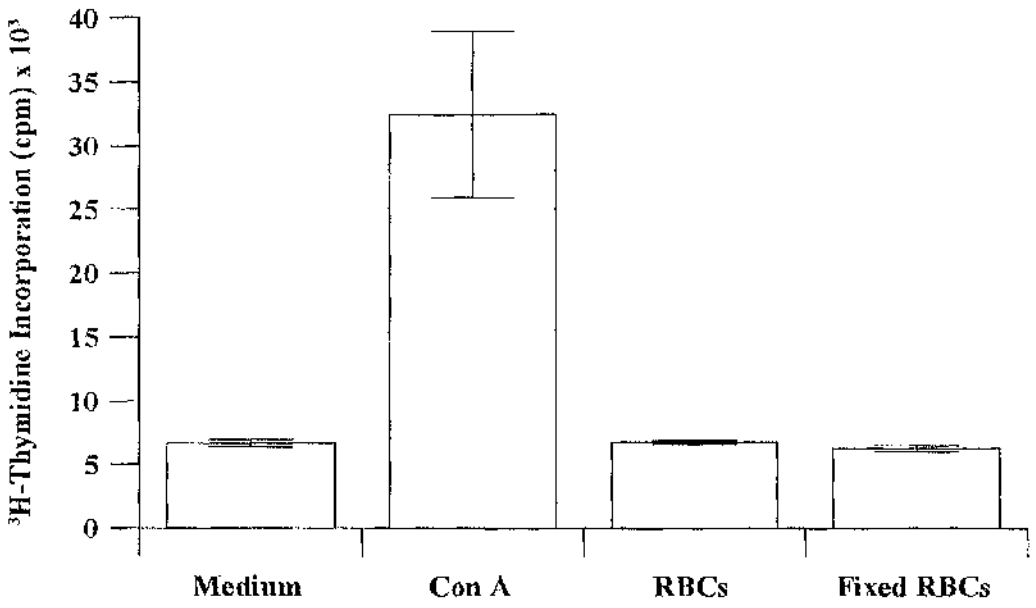


Table 3.1. Immunofluorescence comparison of live, ethanol-fixed and paraformaldehyde-fixed trypanosomes expressing defined antigen types.

VAT	Antibody	Live	Ethanol Fixed	Formaldehyde Fixed
ILTat 1.3	Rabbit anti-1.3	+++	+++	++–
	MAb 23.2	-	+++	-
ILTat 1.3	Rabbit anti-7.1	-	-	-
	MAb 17.2	-	-	-
GUTat 7.1	Rabbit anti-7.1	+++	+++	+++
	MAb 17.2	-	+++	-
GUTat 7.1	Rabbit anti-1.3	-	-	-
	MAb 23.2	-	-	-

The immunofluorescence antibody technique was used to investigate whether the paraformaldehyde fixation process disrupts the trypanosome surface coat and exposes subsurface epitopes of the VSGs. ILTat 1.3 and GUTat 7.1 trypanosomes were used live or ethanol-fixed on smears and compared with smears of these two VATs using paraformaldehyde-fixed trypanosomes. Rabbit anti-ILTat 1.3, for ILTat 1.3, and rabbit anti-GUTat 7.1, for GUTat 7.1, were used as positive controls, monoclonal antibody 23.2 is a VSG-specific subsurface antibody for ILTat 1.3 and monoclonal antibody 17.2 is a VSG-specific subsurface antibody for GUTat 7.1.

Figure 3.4. Paraformaldehdye-induced proliferation by RBCs.



Mononuclear splenocytes were harvested from GUTat 7.2 infected mice after 2 days of infection and stimulated *in vitro*. The cells were stimulated with medium alone, Con A (8μg/ml) or sheep RBCs. The RBCs were used at 2×10^6/ml as were the paraformaldehyde-fixed RBCs. The fixation process was the same as that used to fix the trypanosome VATs. The results are expressed as the means ± 2 SE.

Figure 3.5. Proliferative responses of mononuclear splenocytes from mice uninfected (a) or infected with trypanosomes expressing ILTat 1.3 (b) and harvested at first peak of parasitaemia (day 4 of infection). Splenocytes were cultured *in vitro* for 72 hours and proliferative responses determined by ^{3}H-Thymidine incorporation in response to medium alone, Con A (8μg/ml) or paraformaldehyde-fixed trypanosomes (2×10^{6}/ml) expressing ILTat 1.3 or ILTat 1.61. Assays were conducted in quadruplicate and data are expressed as mean ± 2 SE.

Figure 3.5a. Proliferation of mononuclear splenocytes from uninfected mice.

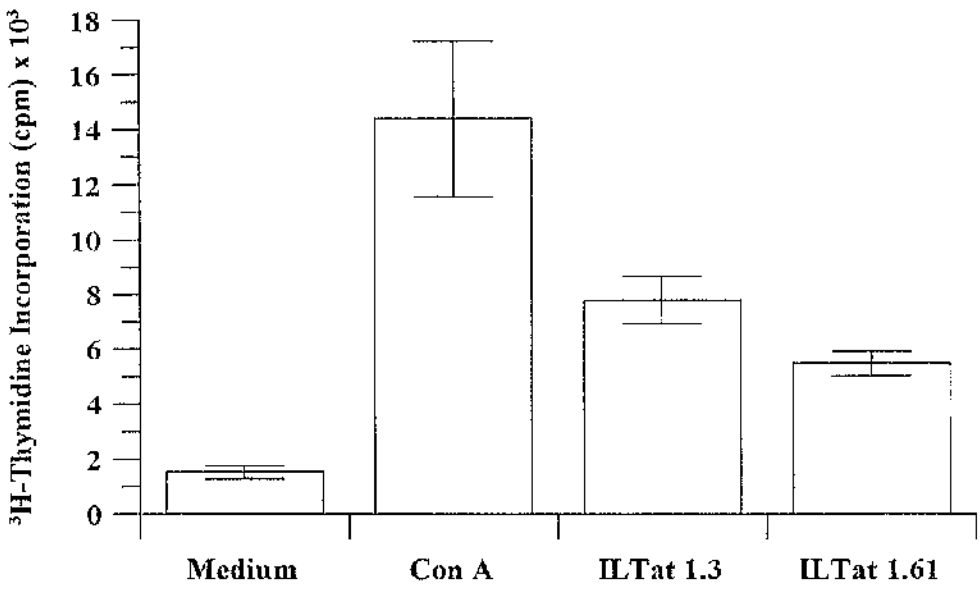
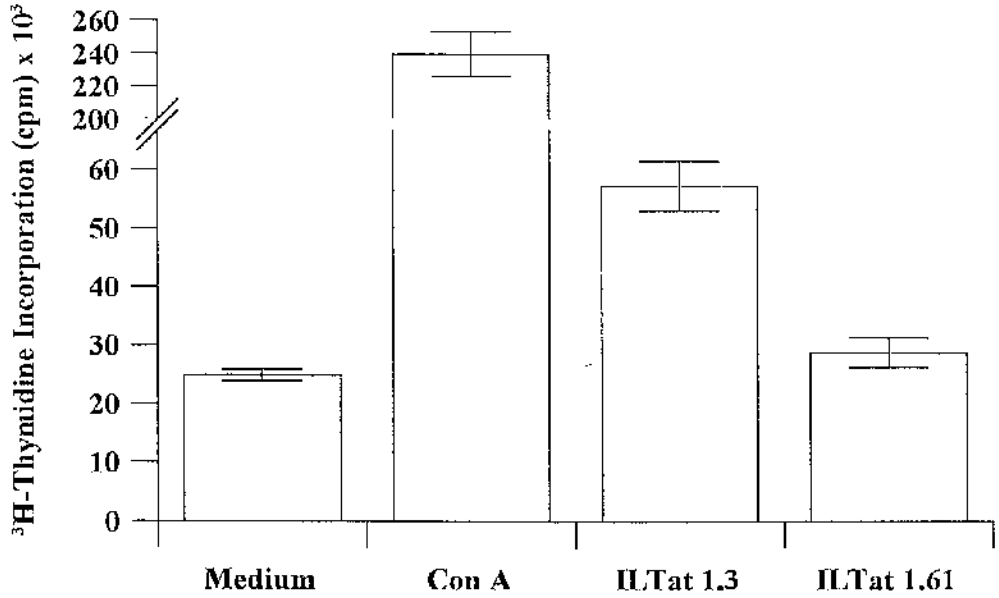


Figure 3.5b. Proliferation of mononuclear splenocytes from ILTat 1.3-infected mice.



CHAPTER 4

CHARACTERISATION OF THE IMMUNE RESPONSE TO ACUTE-PHASE TRYPANOSOME INFECTIONS AND COMPARISON OF RESPONSES TO DIFFERENT INFECTION AND IMMUNISATION REGIMES

4.1. INTRODUCTION

African trypanosomes evade the immune response by antigenic variation thereby changing their highly immunogenic VSG coat to express a different antigen type (Borst & Cross,1982; Turner & Barry,1989; Borst,1991a). They also invoke a profound depression of immune responsiveness, both in cattle (Nantulya *et al*,1982; Sileghem & Flynn,1992) and, particularly, in mice (Dempsy & Mansfield,1983; Sileghem *et al*,1986; Darji *et al*,1993; Stemberg *et al*,1994; Mabbott *et al*,1995). In combination, these processes make it extremely difficult to investigate trypanosome VAT-specific immune responses.

Most studies of effector immune responses to trypanosomes to date have focused on humoral mechanisms (reviewed by Vickerman,1985) but it is still questionable as to whether antibody production is primarily polyclonal in origin (Diffley,1983) or VAT-specific (Musoke *et al*,1981). Almost all studies of antigenic variation have considered it exclusively as a mechanism for evading these humoral responses (see for example Van Meirvenne *et al*,1975).

A few investigations have indicated that T-cell responses are likely to exist (Campbell *et al*,1978; Gasbarre *et al*,1980; Bakhiet *et al*,1993; Schleifer *et al*,1993) and it has recently been suggested that this component of the immune response is likely to be T-helper 1 type in character, but only in the peritoneum (Schleifer *et al*,1993). The major impediment to understanding T-cell biology in the context of a systemic trypanosome infection has, in my view, been the failure to detect antigen-driven proliferative responses from splenocytes, but this shortcoming has now been overcome (Chapter 3). The

principle objective of the work described in this chapter was to provide a basic description of the VAT-specific immune response, both humoral and cell-mediated, in trypanosome-infected mice, at the first peak of parasitaemia when proliferative responses are detectable. The data generated also provided the basic groundwork required to address the question as to whether antigenic variation is a T-cell, as well as an antibody, evasion mechanism.

Immunisation studies using defined antigens have rarely been conducted in African trypanosome research. However, immunisation with irradiated parasites has been shown to engender protective immunity (Wellde *et al*,1975; Pinder *et al*,1986) and VAT-specific passive immunisation achieved using VAT-specific monoclonal antibodies (Crowe *et al*,1984). Non-variant antigens induce detectable antibody responses within ten days in trypanosome-infected mice (Pearson *et al*,1986) and in one report, immunisation with non-variant flagellar pocket antigens provided partial protection (Olenick *et al*,1988). A more recent and careful study has failed to repeat this observation however (Shapiro,1994). There is a larger literature on immunisation employing infection/cure regimes but with antigenically undefined, often tsetse-transmitted, infections (see for example Pinder *et al*,1986). With the benefit of hindsight and in light of our knowledge of the scale of the variant repertoire (Borst & Cross,1982; Barry & Turner,1991; Turner *et al*,1988) and the instability of the metacyclic VAT repertoire (Barry *et al*,1983; Turner *et al*,1988), these observations are almost impossible to interpret. A secondary objective of the work described in this chapter therefore has been to compare VAT-specific immune responses in mice either infected or immunised with antigenically defined trypanosome populations.

4.2. RESULTS

4.2.1. Analyses Of Antibody Responses

Antibody-dependent complement-mediated lysis assays were used to determine the lytic titres of the antibodies present in the plasma of individual mice. These samples were

assayed with live trypanosomes expressing a VAT homologous to that of the initial infection or immunisation, and with a heterologous antigen type. This assay thus provides an index of VAT-specific antibody effector function. All the samples taken from infected mice were unable to cause lysis of heterologous antigen types but were however able to lyse homologous trypanosomes [Table 4.1a.]. Plasma from uninfected mice could not lyse any of the trypanosomes showing that mouse plasma cannot in itself lyse trypanosomes under these conditions. The lytic titres of the plasma samples from the infected mice were the same within each group, resulting in standard errors of zero for each group. When reciprocal titres were compared between groups they differed however and ranged from 4 to 128. This range was not surprising due to the ever expanding trypanosome populations within each infection towards the first peak of parasitaemia.

The samples from the mice infected and drug cured produced VAT-specific lytic titres consistant with a typical IgM response [Table 4.1b.] in that the titres from the peak of parasitaemia were lower, 4, at the first parasitaemic peak and increased to 128 one week after drug cure but decreased to 16 two weeks after drug cure.

Two weeks after a single immunisation with paraformaldehyde-fixed trypanosomes the plasma was assayed. Each VAT used for immunisation produced a significant lytic titre towards the homologous antigen types with each being over 128, the highest level of detection in the assay [Table 4.1c.]. All four groups of immunised mice were unable to cause lysis of the trypanosomes they were assayed against.

These antibody-dependent complement-mediated lysis assays were carried out at least three times.

To investigate the effects of infection on antibody subclass/isotype levels, and the VAT-specificity of these responses, I employed a combination of radioimmunodiffusion and absorption assays. IgM, IgG_1, IgG_{2a} and IgG_{2b} levels in plasma samples from individual mice were measured and found to be identical in all mice within a group. Plasma samples were therefore pooled, reassayed and subjected to repeated rounds of absorption with live trypanosomes of defined VATs before being assayed once more.

The difference in titres between assays before and after absorption represents the VAT surface epitope-specific titre.

Preliminary experiments were conducted to ensure that absorption was being conducted to completion and was VAT-specific. Plasma from GUTat 7.2 and ILTat 1.3-infected mice each revealed that two rounds of absorption was sufficient to determine VSG surface epitope-specific antibodies as there was no reduction in the levels of isotypes after the third and fourth incubation [Table 4.2.]. Control experiments showed that this absorption was VAT-specific: plasma from uninfected mice was mixed with GUTat 7.2 live trypanosome pellets and there was no absorption [Table 4.2.]. Also, when the plasma from ILTat 1.61-infected mice was incubated with GUTat 7.2 trypanosomes there was no absorption [Table 4.2.]. Therefore two rounds of absorption was sufficient to determine the VSG surface epitope-specific isotypes present in the plasma of infected mice.

During infections with each of the four VATs there were increases in the total levels of IgM, IgG_1 and IgG_{2a} when compared with the total immunoglobulin isotypes from the uninfected mice but there was no detectable IgG_{2b} in the samples taken at the peak of parasitaemia [Table 4.3.]. The plasma samples taken from GUTat 7.2-infected mice two days after the peak of parasitaemia showed an increase in the total level of IgM which was far greater than the levels of IgM at the peak of parasitaemia. The total level of IgG_1 was paradoxically reduced and there was no detectable IgG_{2a} or IgG_{2b}.

The results from the antibody-dependent complement-mediated lysis assays suggest that these increases in levels of isotypes are likely to have been caused by the development of VAT-specific antibodies and direct evidence in support of this notion was obtained from absorption assays. Specific IgM antibodies were present at the peak of each infection with highest levels of specific IgM present in the plasma from the mice showing a decline in parasitaemia [Figure 4.1.]. Specific IgG_{2a} antibodies were detected in the samples from the ILTat 1.3 and GUTat 7.2-infected mice but not in the other groups. No specific IgG_{2b} antibodies were detected in any of the samples. The most notable findings were very high levels of IgG_1 antibodies produced in each infection

although the levels were not as high at the decline of parasitaemia compared with those at the peak.

Because plasma samples were analysed from each individual mouse and the antibody-dependent complement-mediated lysis and antibody isotypes showed no difference between the samples within each group, it was considered acceptable for the splenocytes to be pooled for all mouse groups for use in flow cytometry, proliferation and cytokine assays.

4.2.2. Antigen-driven T-cell Proliferative Responses

The proliferative responses of mononuclear splenocytes at first peak of parasitaemia from mice infected with each of four different VATs are shown in Figures 4.2b-e, in comparison to results from uninfected mice [Figure 4.2a.]. In all these cases, proliferative responses from splenocytes of infected mice were raised in the absence of any added stimulant and I attribute these responses to carry-over of antigen partially processed in antigen presenting cells from mice into culture. Despite these raised background levels, enhanced proliferation was clearly seen in response to mitogen (5-10 fold higher than medium controls) and homologous antigen (2.5-4 fold higher). Interestingly, some degree of proliferation driven by heterologous VATs was also seen. In splenocytes from ILTat 1.3-infected mice some heterologous antigen-driven proliferation with GUTat 7.1 and GUTat 7.2 was observed, less with ANTat 1.8 and very little with ILTat 1.61 [Figure 4.2b.]. Some degree of reciprocity in driving of proliferative responses with heterologous VATs was apparent. Strong heterologous responses between ILTat 1.3 and GUTat 7.1 [Figure 4.2d.] were observed and GUTat 7.2 also generated strong responses from splenocytes of mice infected with these VATs. In contrast, ILTat 1.61 generated only poor responses from splenocytes of ILTat 1.3-infected mice [Figure 4.2b.] and vice versa [Figure 4.2c.]. The splenocytes from the ILTat 1.61-infected mice also proliferated to some degree with GUTat 7.1 and GUTat 7.2.

When the mononuclear splenocytes were examined during the decline from the first parasitaemic peak [Figure 4.2f.] the pattern of proliferation in response to mitogen and antigenic stimulation was extremely similar to that produced by the GUTat 7.2-infected mice at peak parasitaemia [Figure 4.2e.]. The proliferative response against homologous and heterologous antigens was only slightly less than responses from mice at the peak of parasitaemia but the response to Con A was approximately 50% less.

Mice infected with ILTat 1.3 were allowed to reach peak parasitaemia before being drug cured and their spleens removed either one week or two weeks after cure. The mononuclear splenocytes one week after cure [Figure 4.3a.] proliferated in response to medium, mitogen and the five VATs used in the assay. The general pattern of response, albeit producing lower ^{3}H-Thymidine incorporation results, was very similar to that of the data from the first peak of infection [Figure 4.2b.] in that there was a strong homologous proliferative response as well as strong heterologous proliferation induced by GUTat 7.1 and GUTat 7.2. The response to stimulation with ILTat 1.61 and ANTat 1.8 was less. The response to medium and Con A was also reduced compared with the data from infected mice although the mitogen response was still several fold greater than the medium and trypanosome antigen induced responses. Two weeks after cure, there was only background levels of proliferation when the cells were stimulated with culture medium alone, varying degrees of heterologous proliferation and a mitogen-induced response which was still several fold greater than the trypanosome induced response [Figure 4.3b.]. The homologous driven proliferation remained the strongest response of the antigen-driven responses even though it was 2-fold lower than that produced one week after cure [Figure 4.3a.] and 5-fold lower than at the first peak of parasitaemia [Figure 4.2b.].

The proliferative responses of mice immunised with paraformaldehyde-fixed trypanosomes was also investigated. The mononuclear splenocytes were harvested two weeks after immunisation with homogeneous trypanosome suspensions. In general, the proliferative responses were several fold lower than those produced by the infections but the responses to stimulation with Con A continued to remain several fold greater than those of the medium controls [Figures 4.4a-d.]. Homologous antigen-driven

proliferation was always the strongest of the antigen-driven responses in each of the four immunisations with varying degrees of heterologous driven proliferative responses.

The proliferation of peritoneal populations from trypanosome-infected mice were also examined at the first peak of parasitaemia. The lymphocytes were counted and dispensed at the same concentration as the assays from the splenocytes, that is 5×10^5 cells/well. This was carried out for all four infections but produced no proliferation in response to medium alone, mitogen or any of the five VATs (data not shown).

4.2.3. Flow Cytometry Analyses

The mononuclear splenocyte populations were examined for all the groups by flow cytometry following cell staining for a number of different cell populations. Using the splenocytes at the first peak of a GUTat 7.1 infection as an example, histograms and dot plots were used to determine the number of cells within each population. These analyses showed that there was no autofluorescence [Figure 4.5a.] and that distinct populations of B-cell, CD3, CD4, CD8, CD25 and NK cells could be determined [Figure 4.5b-g].

When comparing the cell numbers/spleen of each of four groups of mice at first parasitaemic peak of a *T.brucei* infection, alterations in the abundances and proportions of mononuclear splenocytes were observed when compared with splenocytes from uninfected mice [Figure 4.6a. & Table 4.4a.]. The numbers of B-cells increased several fold during infection as would be expected given the prominent antibody response during trypanosome infections. The numbers of T-cells per spleen also increased markedly and this increase was seen in both the CD4 and the CD8 cell populations despite decreases in the percentages of CD3 and CD4 cells and only negligible increases in the proportions of CD8 cells. Numbers of NK cells decreased at first peak of parasitaemia both in abundance and relative proportion. The ratio of B:T cells increased during infection but the ratio of CD4:CD8 cells decreased resulting from a greater increase, in relative terms, of the CD8 compared with the CD4 population [Table 4.4a.]. The ratios of activated cells (using CD25 as a marker of activation) also altered indicating an increase in the proportions of both activated CD4 and activated CD8 cells during infection.

The cell abundances of mononuclear splenocytes from the four groups of immunised mice differed between each group [Figure 4.6b.]. As shown in the infected mice, the numbers of B and T-cells per spleen increased several fold as did the numbers for both the CD4 and CD8 populations. Nevertheless, the percentage of B-cells did not alter significantly when compared with the uninfected control mice [Table 4.5b.]. The percentage of T-cells from the ILTat 1.61 and GUTat 7.2 immunised mice decreased, resulting in an increase in the B:T cell ratio, compared with the other 2 groups of immunised mice [Table 4.5b.]. The CD4:CD8 ratios of the immunised mice were more similar to the ratios shown from the infected mice than those of the uninfected mice but the numbers of activated CD4 or CD8 cells present in the immunised mice were lower than in the infected mice.

When comparing the cell numbers per spleen it was noticeable that there were more T-cells present in each of the spleens from the immunised mice compared to the numbers present in the infected mice [Figures 4.6a-b.] and this was observed, with the exception of the ILTat 1.3 CD8 cells, in both the CD4 and CD8 populations.

4.2.4. Cytokine Production.

High levels of IFNγ (more than 1500U/ml) were detected in the cytokine assay supernatants from mononuclear splenocytes of ILTat 1.3 infected mice, at the first peak of parasitaemia, when stimulated *in vitro* with Con A [Figure 4.7a.]. This was the case when supernatants were harvested at both 24 and 72 hours. IFNγ was also detected in those supernatants which were stimulated with paraformaldehyde-fixed trypanosomes of the homologous and the four heterologous VAT's. Similar results were observed using splenocytes from ILTat 1.61 [Figure 4.7b.], GUTat 7.1 [Figure 4.7c.] and GUTat 7.2 [Figure 4.7d.] infected mice in that high levels of IFNγ were produced in response to Con A and there was a marked response to stimulation with the homologous VAT and with some of the heterologous VATs.

The levels of detectable IFNγ differed after the first parasitaemic peak; compare Figure 4.7d. with Figure 4.8. The response after stimulation with Con A was several

fold lower two days after peak than that at peak with levels less than 240U/ml compared with greater than 1500U/ml. IFNγ was produced in response to homologous and heterologous VATs but the levels produced by stimulation with medium alone in the 72 hours supernatant were high compared with the IFNγ production from the antigen stimulated wells.

There was no homologous or heterologous antigen-driven IFNγ production from the mice infected and killed one week or two weeks after drug cure. There was however production of IFNγ when the mononuclear splenocytes were stimulated with Con A. Both groups of mice produced more than 1500U/ml, where 1500U/ml was the top standard of the assay, after 24 and 72 hours of stimulation *in vitro* (data not shown).

In immunised mice there was no detectable IFNγ after *in vitro* stimulation of mononuclear splenocytes with homologous or heterologous fixed trypanosomes from any of the four groups of immunised mice. IFNγ was produced after mitogen stimulation of the splenocytes from each of the four groups [Figure 4.9.] although these levels were extremely low compared to the levels produced by splenocytes from infected mice after Con A stimulation.

No significant levels of IL-4 (<0.5U/ml), IL-5 (<1U/ml) or IL-6 (<1U/ml) were produced during stimulation of mononuclear splenocytes from ILTat 1.3, ILTat 1.61, GUTat 7.1 and GUTat 7.2 infected mice, at the first peak of parasitaemia or two days after peak, in response to mitogen or fixed parasites expressing homologous or heterologous trypanosome VATs. The levels of IL-4, IL-5 and IL-6, were also investigated during a time course experiment and there were no detectable levels of any of these cytokine above 1U/ml at any of the time points examined (data not shown).

In combination, these observations of a proliferative response with production of IFNγ but no IL-4, IL-5 or IL-6 are indicative of a T-helper 1 type response and there was therefore a reasonable expectation to observe a substantive IL-2 response. However, only low levels of IL-2 were detected using splenocytes from mice at the first peak of parasitaemia compared to splenocytes from uninfected mice when stimulated with Con A [Figure 4.10a.] and there was no trypanosome-driven IL-2 production (data not shown). This was observed in four different infections. At the decline from the first

peak of parasitaemia there was significantly lower IL-2 levels even than those detected at the peak of infection. A separate set of experiments was therefore conducted to investigate the time course of potential IL-2 production which revealed that there was a substantive production of IL-2 but it occurred very early during infection and had almost waned by the time of first parasitaemic peak of infection - 96 hours [Figure 4.10b.]. This was observed in infections of both ILTat 1.3 and GUTat 7.1.

These was no detectable IL-2 produced from mice either one week or two weeks after drug cure of infection when stimulated with homologous or heterologous VATs and only very small amounts of IL-2 production after Con A stimulation [Figure 4.11a.] - no more than 8U/ml for the one week samples and no more than 12U/ml from the splenocytes purified two weeks after cure. Con A stimulation resulted in low levels (<18U/ml) of IL-2 production with the mononuclear splenocytes from each of the four groups of immunised mice [Figure 4.11b.] but there was no detectable IL-2 produced when stimulated with paraformaldehyde-fixed trypanosomes.

Cytokine ELISAs for IL-2, IL-4, IL-5, IL-6 and IFNγ were also conducted using peritoneal cell populations from the four groups of mice at the first peak of parasitaemia. No cytokine production was detectable in any samples tested either at the 24 hour or 72 hour harvest.

4.3. DISCUSSION

The antibodies tested during my experiments were able to specifically kill the infecting trypanosome VAT but were unable to cause lysis of heterologous VATs. The antibodies produced were therefore VAT-specific and produced in response to the particular invading antigenic type as has been previously shown (Musoke *et al*,1981; Sendashonga & Black,1982). These data conflict with the suggestion that the B-cells in trypanosome-infected mice are non-specifically activated and that some of the antibodies are not specific for the trypanosome VAT (Gasbarre *et al*,1981; Diffley,1983).

At the first peak of infection, the plasma samples showed an increase in levels of IgM, IgG_1 and IgG_{2a} but I failed to detect any IgG_{2b}. Of these immunoglobulin isotypes, I detected varying amounts of VSG surface epitope-specific IgM, IgG_1 and IgG_{2a}, in some cases, which contrasts with other studies. These other studies have claimed that during infections with monomorphic lines of trypanosomes, such as those employed here, there is no B-cell activation and therefore no antibody production, which in turn relates to these mice being unable to control the first peak of parasitaemia (Sendashonga & Black,1982; Black *et al*,1986; Sendashonga & Black,1986). High levels of specific IgM, IgG_1 and IgG_2 were detected in trypanosome-infected cattle but the IgG antibodies were detected one week after the IgM (Masake *et al*,1983). In another study bovine IgM and IgG_1 antibodies were detected which were VAT-specific but they failed to detect any IgG_2 (Musoke *et al*,1981). In a previous study in mice IgM, IgG_1, IgG_{2a} and IgG_{2b} responses have all been detected (Sendashonga & Black,1982)

VAT-specific splenic T-cell responses do occur during acute *T.brucei* infections and these responses are in the form of T-cell proliferation, using the assay system developed in Chapter 3, and IFNγ production. Although there was no antibody response detected to heterologous VATs there was a degree of heterologous antigen-driven proliferation in some cases but not in others. These observations suggest that the highly sophisticated antigenic variation process shown by the trypanosomes may possibly be a mechanism to evade the host T-cell response as well as the classic evasion of the B-cell response. If evasion of the T-cell response is successful during infection then there would be a lack a T-cell help for the B-cell response which may result in a less efficient antibody response and failure to eliminate the invading trypanosomes thus promoting continuation of the infection. In contrast, Schleifer *et al* (1993) found no T-cell proliferation of splenocytes, peritoneal or lymph node cells when stimulated with purified VSG or with whole cell extracts *in vitro* using cells from mice which had been infected for two weeks. This is possibly due to the use of different mouse or trypanosome strains and the duration of the infection or perhaps because soluble VSG is reported to be less immunogenic than membrane-bound VSG (Diffley,1985).

T-cells and NK cells are probably responsible for the production of high levels of

IFNγ although the numbers of NK cells decreased at the first parasitaemic peak. In another study, depletion of CD8 cells from trypanosome-infected rats abrogated the production of IFNγ which in turn increased survival time (Bakhiet *et al*, 1990). This result may however be caused indirectly by inhibiting immunosuppression; lowering IFNγ levels would reduce numbers of macrophages activated which will lead to improved T-cell competence and killing of more parasites resulting in increased survival time. The highly increased amounts of IFNγ produced during infection may boost the macrophage activity by upregulating surface receptors and therefore increasing phagocytic activity, which is important for removing the trypanosomes from the circulation (Askonas, 1985). IFNγ, in synergy with IL-2, can enhance the synthesis of light chains of immunoglobulins (Romagnani *et al*, 1986) and may aid the host in fighting the pathogens by way of the humoral response. In addition, the activity of the cytotoxic T-cells can be increased due to the upregulation of class I MHC molecules on the cell surface which are induced by IFNγ. These findings may result in the host producing a more effective immune response to the parasites. I would suggest that IFNγ is the major, or at least one of the major, immunoregulatory molecules and it can result in both positive and negative effect for the host in that, during *T.brucei* infections some IFNγ is necessary to stimulate the T-helper 1 response but too much IFNγ can induce immunodepression of the host immune response, at least in the murine model system. In cattle however, the depletion of CD8 cells *in vivo* had no effect on the parasitaemia (Sileghem & Naessens, 1995).

When T-cells are stimulated by Con A or antigens then they produce IL-6. This IL-6 can act on B-cells to induce differentiation and immunoglobulin production. In my system there was no detectable IL-6 when the cells were stimulated with Con A or with antigen but some IL-6 production might reasonably be expected due to the importance of the antibody response. Time course experiments revealed that even before the first peak of parasitaemia there was no detectable IL-4, or IL-5 or IL-6, above 1U/ml at any of the time points examined. During the acute infections, the lack of detectable IL-4, IL-5 or IL-6 is probably due to the inhibition of this response caused by IFNγ therefore resulting in a T-helper 1 type response.

I, like many others, failed to detect much IL-2 at the first peak of parasitaemia (Sileghem *et al*,1989; Sileghem *et al*,1991; Sileghem & Flynn,1992; Darji *et al*,1993; Schleifer & Mansfield,1993). IL-2 production was only detected after mitogen stimulation with Con A and even these levels were far lower than those of the uninfected controls [Figure 4.10b.]. This seemed confusing given the high levels of proliferation exhibited and the greatly elevated IFNγ response. On closer examination, by way of a time course experiment, large amounts of IL-2 were detected but only early in infection and these had dissipated towards the first peak of parasitaemia. These low levels of IL-2 at the first parasitaemic peak could be a result of an increase in IL-2 receptor expression, as shown on both the CD4 and CD8 cells from trypanosome-infected mice, which allowed more IL-2 to bind and therefore not be detected free in the supernatant. After the initial activation, a different cytokine, like IL-12, may be continuing the stimulation of the cells and replacing the IL-2 role. The cells have to be activated in order to respond to IL-12, which will be the case during a *T.brucei* infection, and only extremely small amounts are required. My data are consistent therefore with a classic T_H1 type response in acute-phase trypanosomiasis and are thus at some degree of variance with these reported by Schleifer *et al* (1993) who detected IFNγ produced by splenocytes from infected mice but no IL-2 or IL-4. Similarly no IL-2 or IL-4 was produced by lymph node cells stimulated by purified VSG but the peritoneal population produced IFNγ and IL-2, but not IL-4, in a VSG-specific manner even though there was no proliferative response. An impairment of IL-2 production by splenocytes has been shown during *T.cruzi* (Tarleton *et al*,1984) and *L.donovani* (Reiner *et al*,1983) infections stimulated with Con A.

The spleens from infected mice were clearly increased in size 2-3 times compared to the spleens from uninfected mice by visual inspection during dissection to prepare mononuclear cell populations. These observations was also described by Masake & Morrison (1980) who observed that the spleen returned to normal size in the later stages of infection. An increase in size is expected due to the nature of the infection but what cell types were increasing was unknown. The increase could have been due to B-cells which were non-specifically activated (Gasbarre *et al*,1981). Although the number of B-

cells increased dramatically in the infected spleens, the number of T-cells also increased several fold and this increase could be seen in both the CD4 and CD8 populations. The increase in T-cells could be in the context of B-cell help but more likely as an effective arm of the host immune response which will clear the infection. The proportion of CD4:CD8 T-cells also changed suggesting an important role for T-cells in a trypanosome infection. The increase in CD8 T-cells to 25% of the splenic T-cell population may be of significant importance and could be the cause of the high levels of IFNγ production. The increase in the proportions of activated CD4 and CD8 cells will be beneficial to the host in fighting infection but this contrasts with the claim of Bakhiet *et al* (1993) that the CD8 cells are activated but the CD4 cells are not during *T.brucei* infections.

The numbers of B-cells and T-cells increased several fold in the splenocyte populations from the immunised mice compared to those of the splenocytes from the uninfected control mice but, the percentages of these cell types remained relatively constant indicating that trypanosomes do not only stimulate B-cells but CD4 and CD8 T-cells too. Moreover, the CD4:CD8 ratio was lower in the immunised mice compared to the control therefore showing that there was an increase in CD8 cells during infection as well as immunisation. Together these data suggest the importance of the T-cells in relation to immune response to African trypanosomes. The general pattern of proliferation between the immunised mice and their infected counterparts was extremely similar when each was compared but the IFNγ production differed a great deal.

In conclusion, during acute *T.brucei* infections there are VSG-specific T-cell proliferative responses and production of large amounts of IFNγ. There is, however, a lack of IL-2 and no detectable IL-4, IL-5 or IL-6 production at the first peak of parasitaemia. These data suggest that the *T.brucei* trypanosomes benefit more than initially thought from undergoing antigenic variation. Not only do they evade the B-cell responses but they also perhaps escape from or reduce the competence of T-cell responses. Therefore antigenic variation may also be a T-cell evasion mechanism as well as the classic B-cell evasion mechanism. Whether this T-cell response directly affects the course of a trypanosome infection or functions indirectly by way of generating a more efficient and effective B-cell response has yet to be investigated.

Table 4.1. Antibody-dependent complement-mediated lysis assays were used to determine the lytic titres of plasma samples from mice infected or immunised with trypanosome populations expressing defined VATs. These samples were obtained at the first peak of parasitaemia from infected mice (**a**), from mice drug cured at first parasitaemic peak with samples taken either one or two weeks later (**b**) and from mice immunised with 10^6 paraformaldehyde-fixed trypanosomes (**c**). Mice were individually assayed in groups of five for each experiment. Titres were identical in all mice within each group in every case. 'Mono' refers to a monomorphic trypanosome line whereas 'pleo' refers to a pleomorphic line. Samples taken at 'decline' were taken two days after the first parastiaemic peak.

Table 4.1a. Complement-mediated lysis assay: reciprocal titres of plasma samples from trypanosome-infected mice.

INFECTION	TRYPANOSOMES	RECIPROCAL LYTIC TITRE
Uninfected Control	ILTat 1.3	0
	GUTat 7.2	0
ILTat 1.3	ILTat 1.3	4
	GUTat 7.1	0
ILTat 1.61	ILTat 1.61	128
	GUTat 7.1	0
GUTat 7.1	GUTat 7.1	32
	ILTat 1.3	0
GUTat 7.2 (mono)	GUTat 7.2	64
	ILTat 1.61	0
GUTat 7.2 (pleo) Decline	GUTat 7.2	128
	ILTat 1.61	0

Table 4.1b. Lytic titres of ILTat 1.3 drug cured mice.

SAMPLE	TRYPANOSOMES	RECIPROCAL LYTIC TITRE
Drug cure 1 week	ILTat 1.3	128
	ILTat 1.61	0
Drug cure 2 weeks	ILTat 1.3	16
	ILTat 1.61	0

Table 4.1c. Lytic titres from the plasma of immunised mice.

SAMPLE	TRYPANOSOMES	RECIPROCAL LYTIC TITRE
ILTat 1.3 Immunisation	ILTat 1.3	>128
	GUTat 7.1	0
ILTat 1.61 Immunisation	ILTat 1.61	>128
	GUTat 7.1	0
GUTat 7.1 Immunisation	GUTat 7.1	>128
	ILTat 1.3	0
GUTat 7.2 Immunisation	GUTat 7.2	>128
	ILTat 1.61	0

Table 4.2. Two different plasma samples, GUTat 7.2 and ILTat 1.3, were allowed to absorb onto trypanosome pellets homologous to those from the infections. Four rounds of absorption were carried out and samples from each were isotyped to determine the necessary numbers of rounds of absorption to ascertain VSG surface epitope-specific antibody levels. Plasma from uninfected mice and from ILTat 1.61-infected mice were incubated with GUTat 7.2 trypanosomes to investigate any non-specific absorption which may occur. IgG_{2b} levels were also investigated but there were no detectable antibodies of this isotype detected by this assay system.

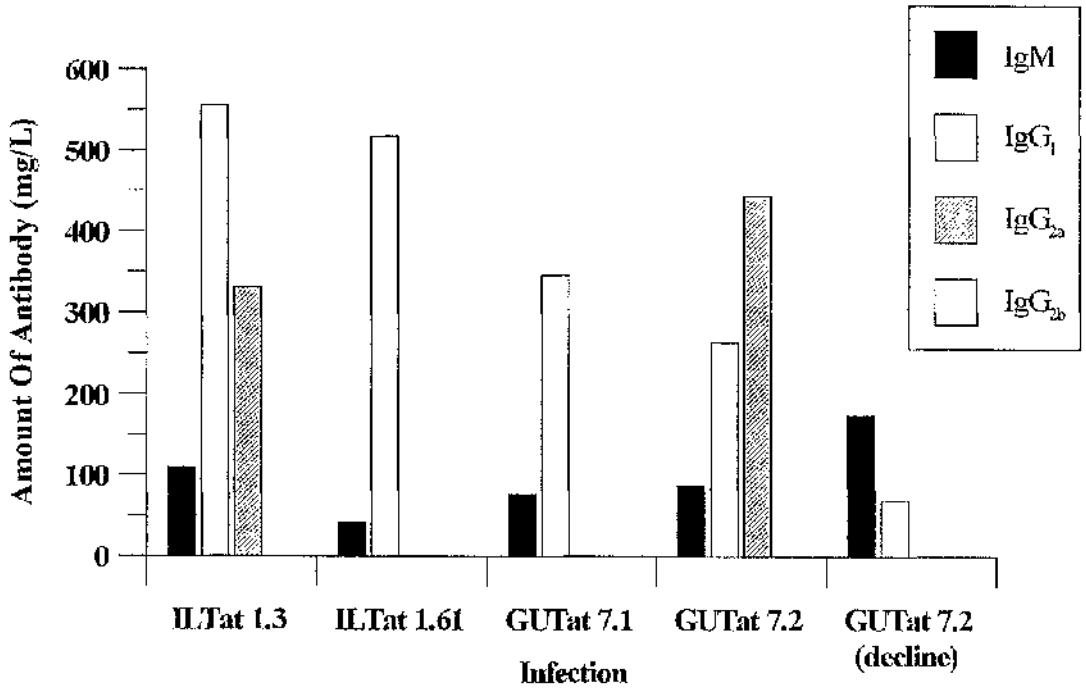
Table 4.2. The effect of multiple rounds of absorption with live trypanosomes expressing defined VATs in IgM, IgG_1 and IgG_{2a} titres in plasma from trypanosome-infected mice.

VAT specificity of plasma	Trypanosome VAT used for absorption	Number of rounds of absoprtion	Antibody isotype/subclass (mg/L)		
			IgM	IgG_1	IgG_{2a}
GUTat 7.2 (Decline)	GUTat 7.2	0	361	323	0
	"	1	198	288	0
	"	2	188	254	0
	"	3	188	254	0
	"	4	188	254	0
ILTat 1.3	ILTat 1.3	0	230	810	923
	"	1	139	288	592
	"	2	121	254	592
	"	3	121	254	592
	"	4	121	254	592
ILTat 1.61	GUTat 7.2	0	112	1420	837
	"	1	112	1420	837
	"	2	112	1420	837
Uninfected control	GUTat 7.2	0	95	472	0
	"	1	95	472	0
	"	2	95	472	0

Table 4.3. Antibody isotype levels of trypanosome-infected mice at the first peak of parasitaemia.

INFECTION	IgM	IgG_1	IgG_{2a}	IgG_{2b}
Control	95	472	0	0
ILTat 1.3	230	810	923	0
ILTat 1.61	112	1420	837	0
GUTat 7.1	139	634	592	0
GUTat 7.2 (mono)	158	551	1480	0
GUTat 7.2(decline)	361	323	0	0

Figure 4.1. VSG surface epitope-specific isotype levels in plasma samples from trypanosome-infected mice at first peak of parastiaemia, except where indicated two days after first peak.



Total antibody isotype levels of the plasma samples from trypanosome-infected mice were determined by radioimmunodiffusion. The plasma samples were then subjected to two rounds of homologous VAT-specific absorption and assayed. The difference in value in each case between the first and second assay provided VSG surface epitope-specific isotype levels. 'Mono' refers to a monomorphic trypanosome line whereas 'decline' is two days after the first peak of parasitaemia of a pleomorphic line.

Figure 4.2. Proliferative responses of mononuclear splenocytes from trypanosome-infected mice and from uninfected mice. The splenocytes were cultured in the presence of culture medium alone, Con A or paraformaldehyde-fixed trypanosomes expressing one of five different VATs. Splenocytes were harvested from uninfected mice (**a**), from mice infected with ILTat 1.3 (**b**), ILTat 1.61 (**c**), GUTat 7.1 (**d**), GUTat 7.2 (**e**), at the first peak of parasitaemia, and from GUTat 7.2-infected mice two days after peak parasitaemia (**f**). Results are expressed as the geometric means ± 2 SE, n = 4.

Figure 4.2a. Proliferation of mononuclear splenocytes from uninfected mice.

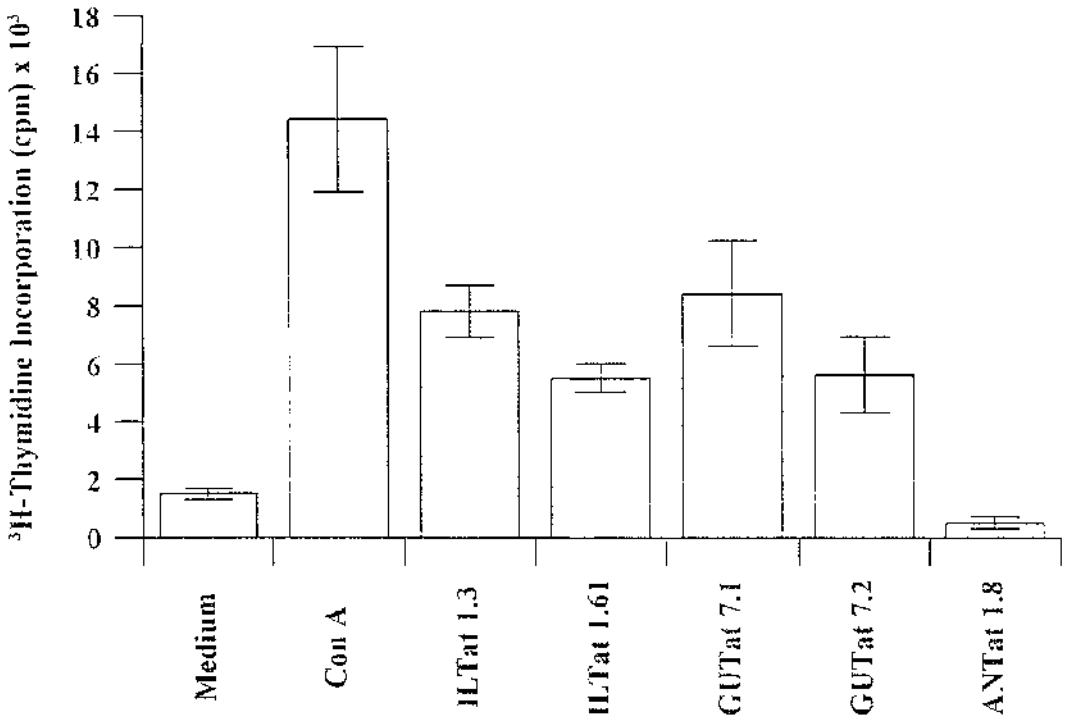


Figure 4.2b. Mononuclear splenocyte proliferation using ILTat 1.3-infected mice.

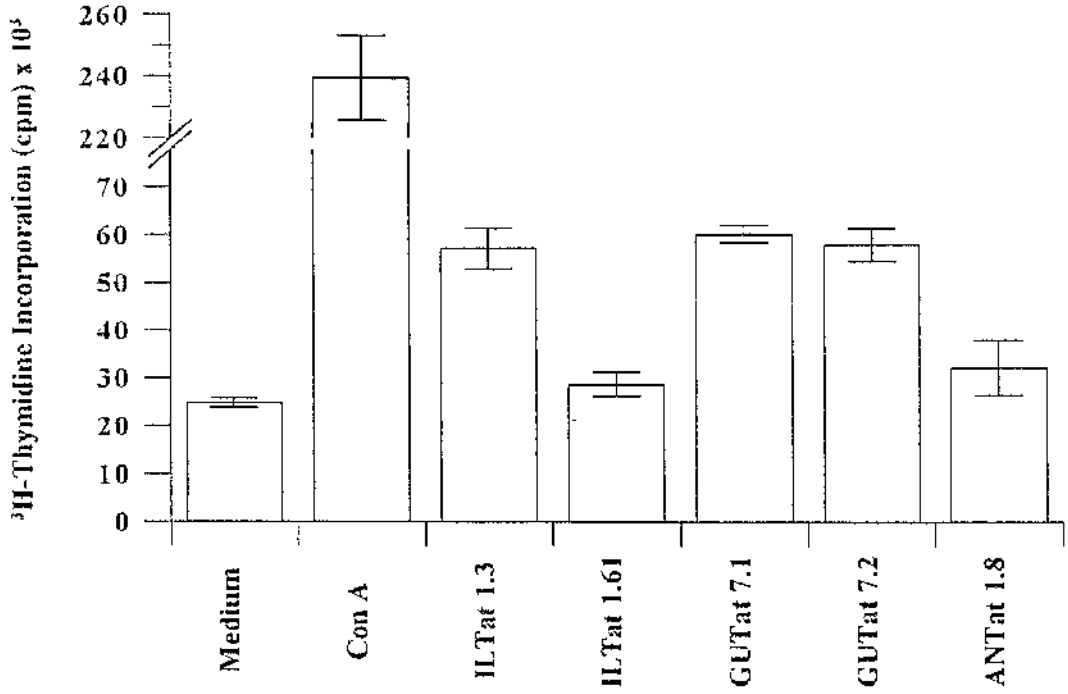


Figure 4.2c. Mononuclear splenocyte proliferation using ILTat 1.61-infected mice.

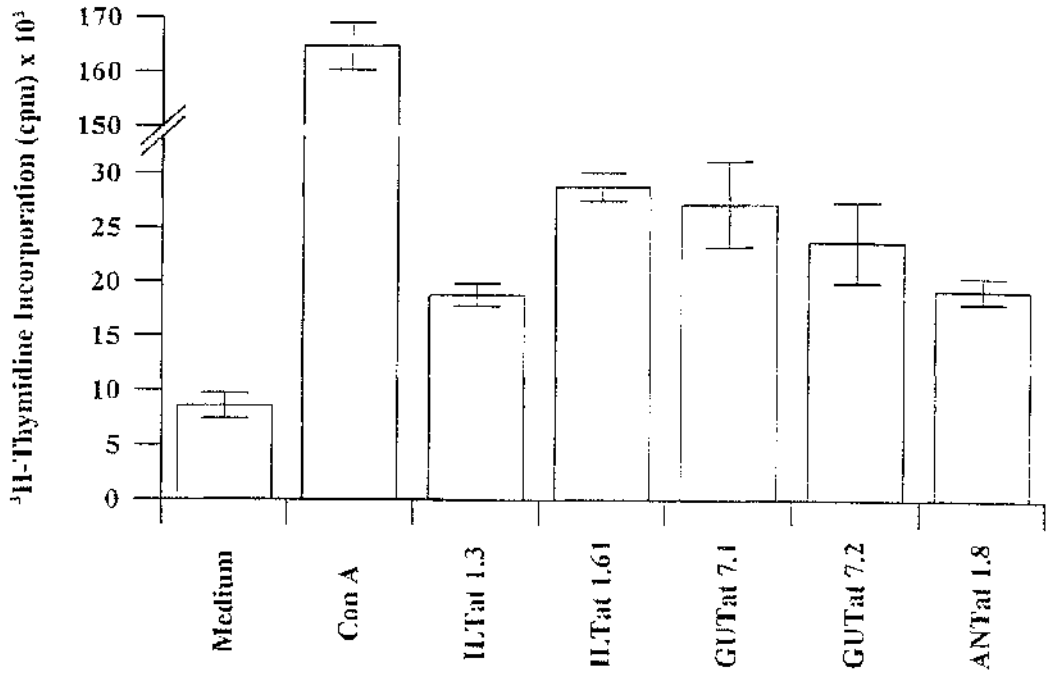


Figure 4.2d. Proliferation of mononuclear splenocytes from GUTat 7.1-infected mice.

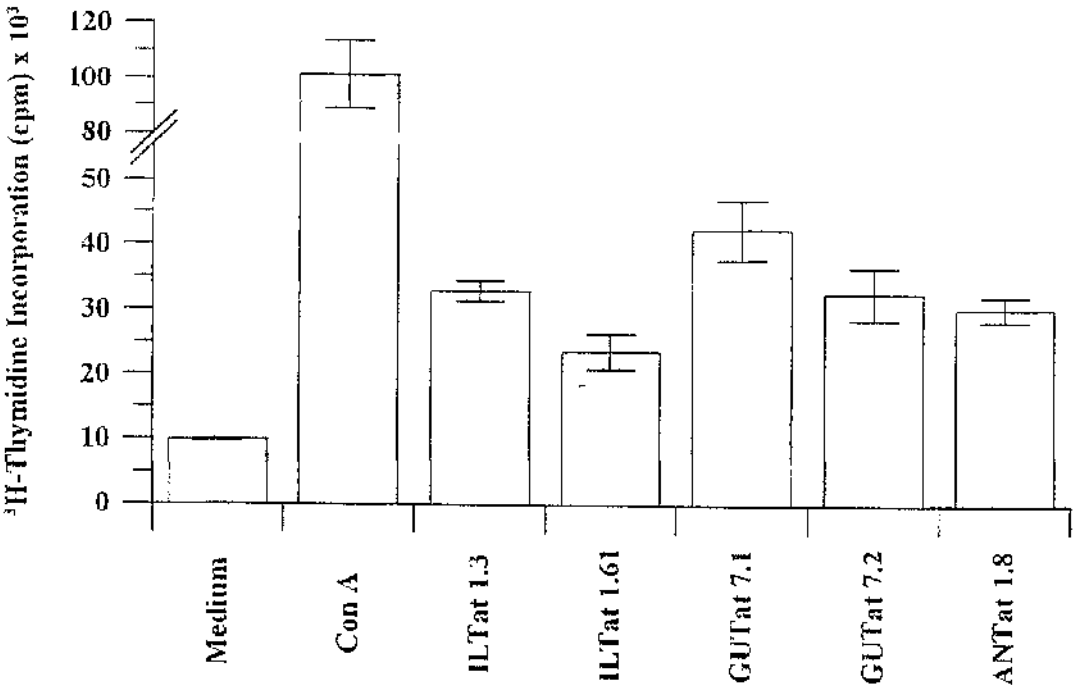


Figure 4.2e. Mononuclear splenocyte proliferation from GUTat 7.2-infected mice at the first peak of parasitaemia.

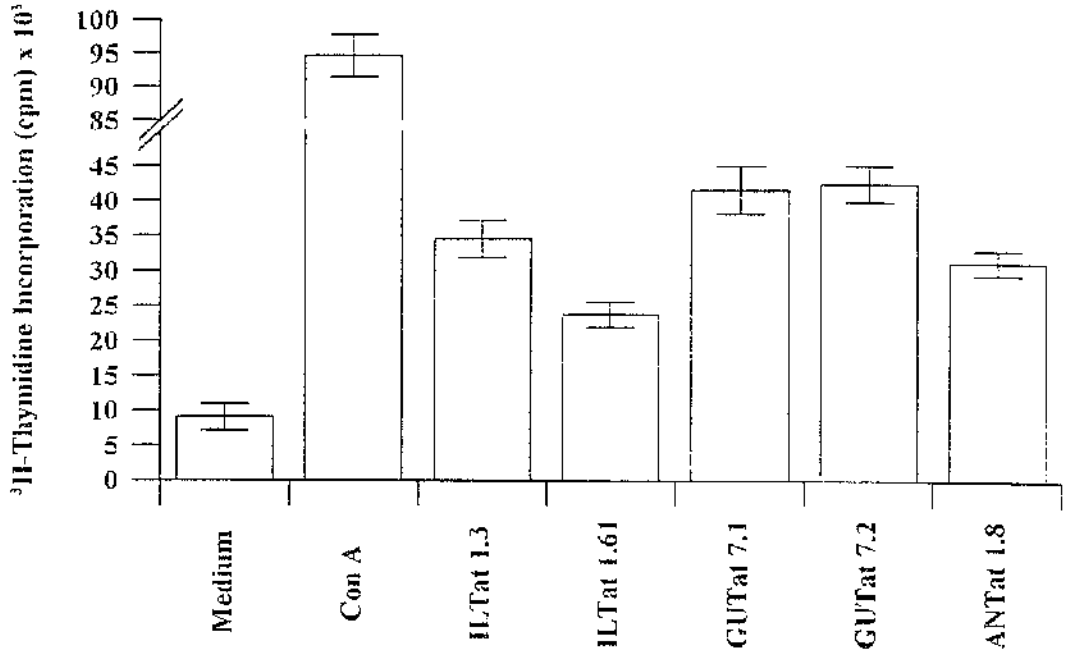


Figure 4.2f. Proliferation of mononuclear splenocytes at the decline of a GUTat 7.2 infection.

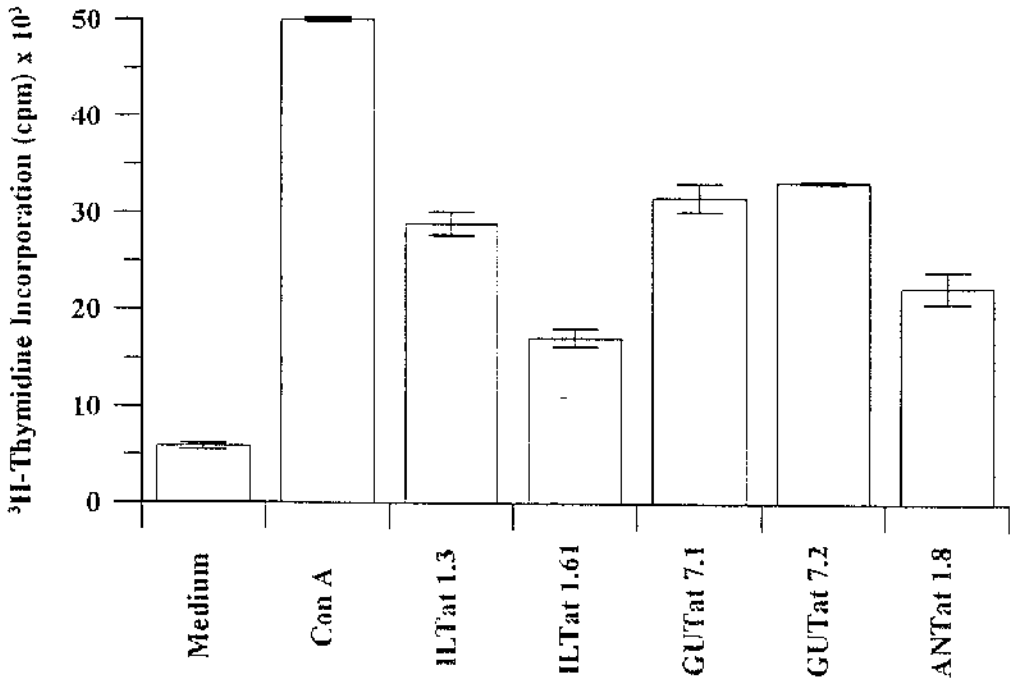


Figure 4.3. Proliferative responses of mononuclear splenocytes from mice infected with ILTat 1.3, drug cured with Cymelarsan at the first peak of parasitaemia and killed either one week (**a**) or two weeks (**b**) later. The cells were stimulated *in vitro* with culture medium, Con A (8μg/ml) or paraformaldehyde-fixed trypanosomes expressing one of five of different antigen types. The means of quadruplicate wells are expressed ± 2 SE, n = 4.

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Figure 4.3a. Mononuclear splenocyte proliferation one week after drug cure.

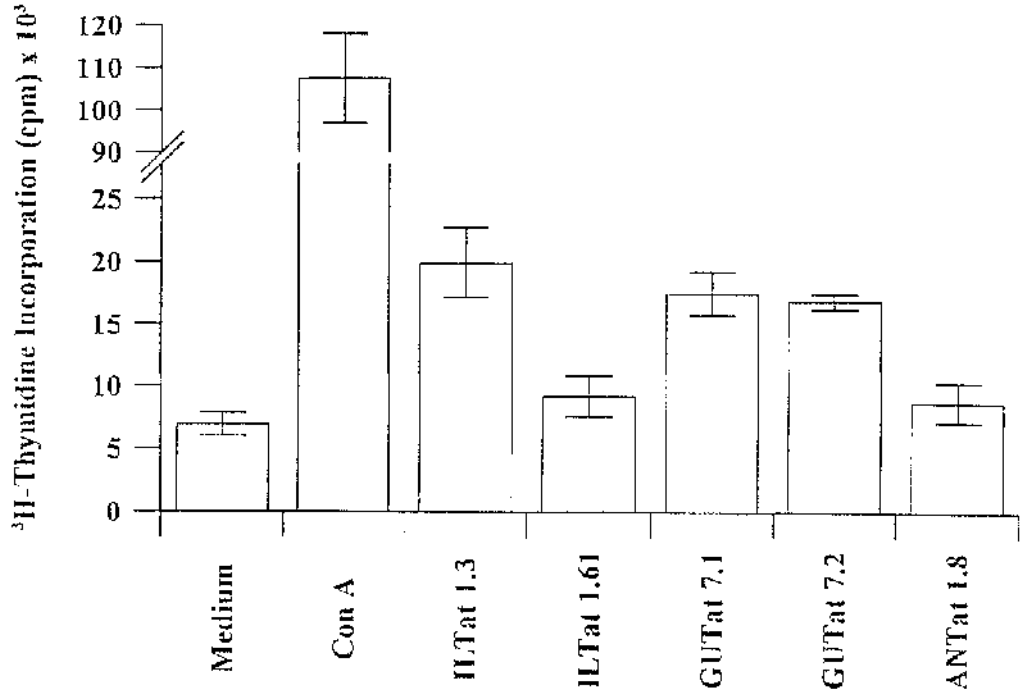

Figure 4.3b. Mononuclear splenocyte proliferation two weeks after drug cure.

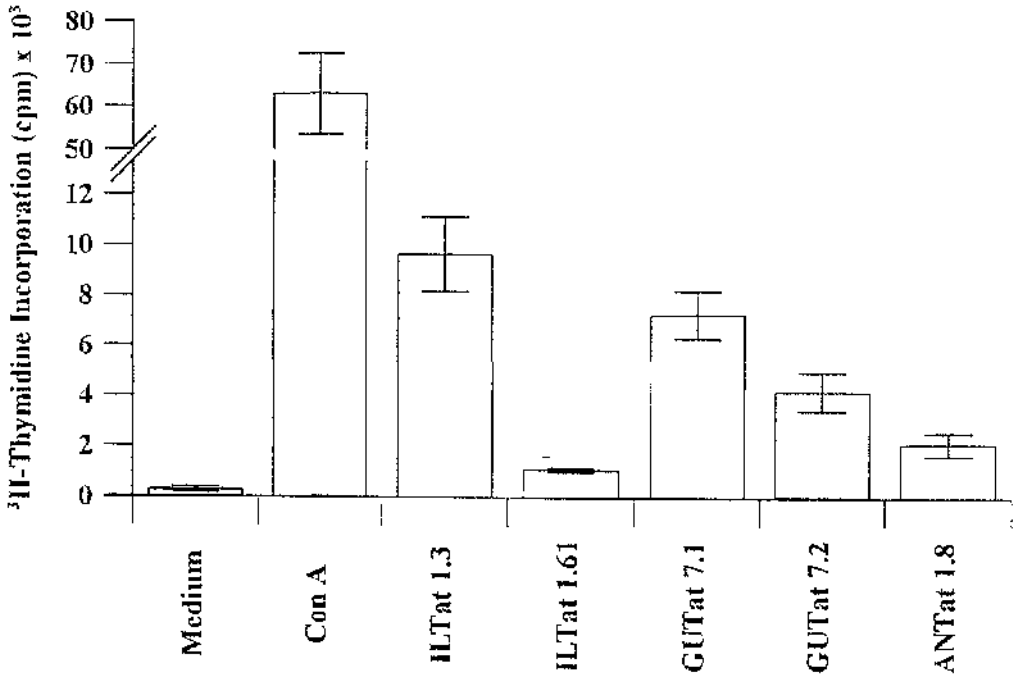

Figure 4.4. Mice were immunised with 10^6 paraformaldehyde-fixed trypanosomes, killed two weeks later and the proliferative responses of the mononuclear splenocytes towards medium alone, Con A or paraformaldehyde-fixed trypanosomes expressing one of five different VATs were determined. Splenocytes were harvested from mice immunised with ILTat 1.3 (**a**), ILTat 1.61 (**b**), GUTat 7.1 (**c**) or GUTat 7.2 (**d**). Results are expressed as the geometric means $\pm$ 2 SE, n = 4.

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Figure 4.4a. Mononuclear splenocyte proliferation after ILTat 1.3 immunisation.

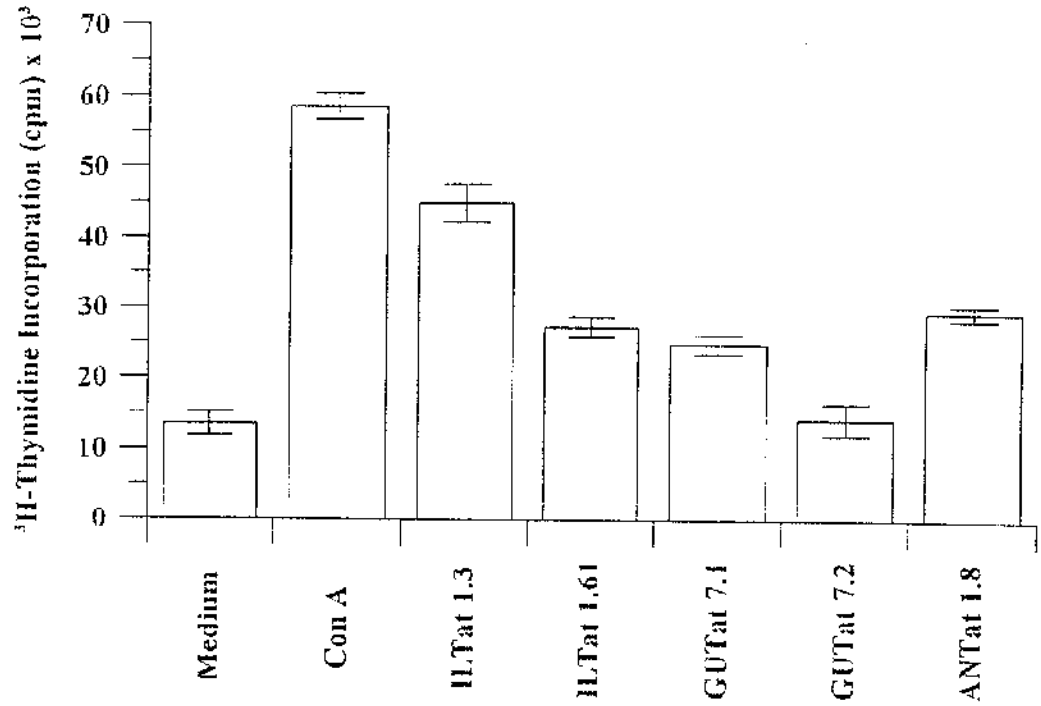

Figure 4.4b. Mononuclear splenocyte proliferation after ILTat 1.61 immunisation.

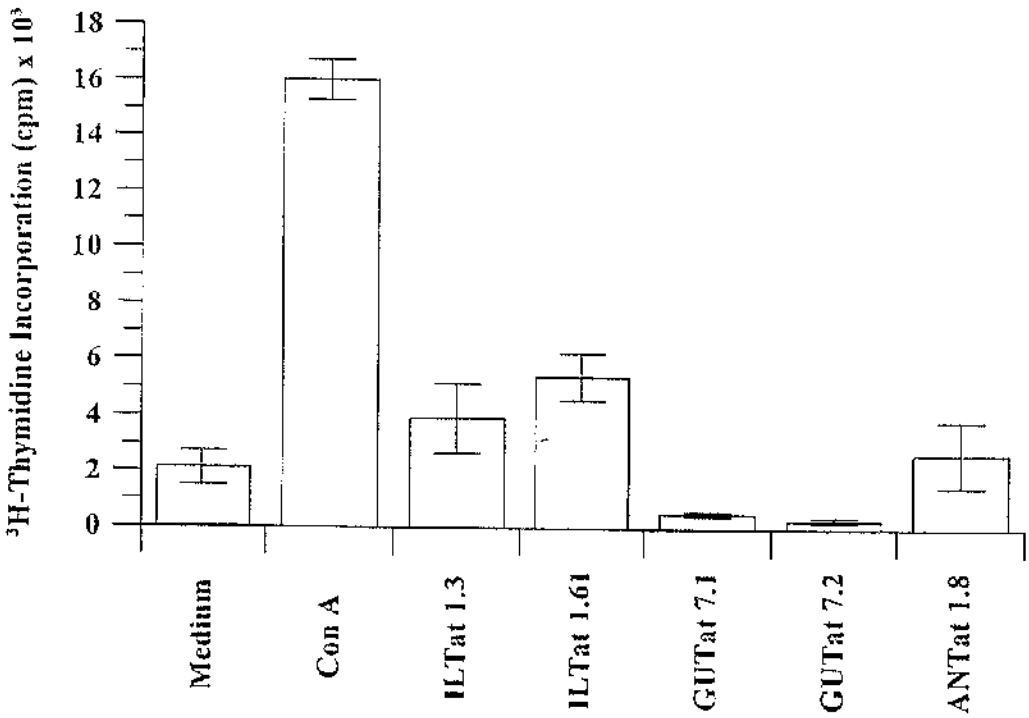

Figure 4.4c. Proliferation of mononuclear splenocytes after GUTat 7.1 immunisation.

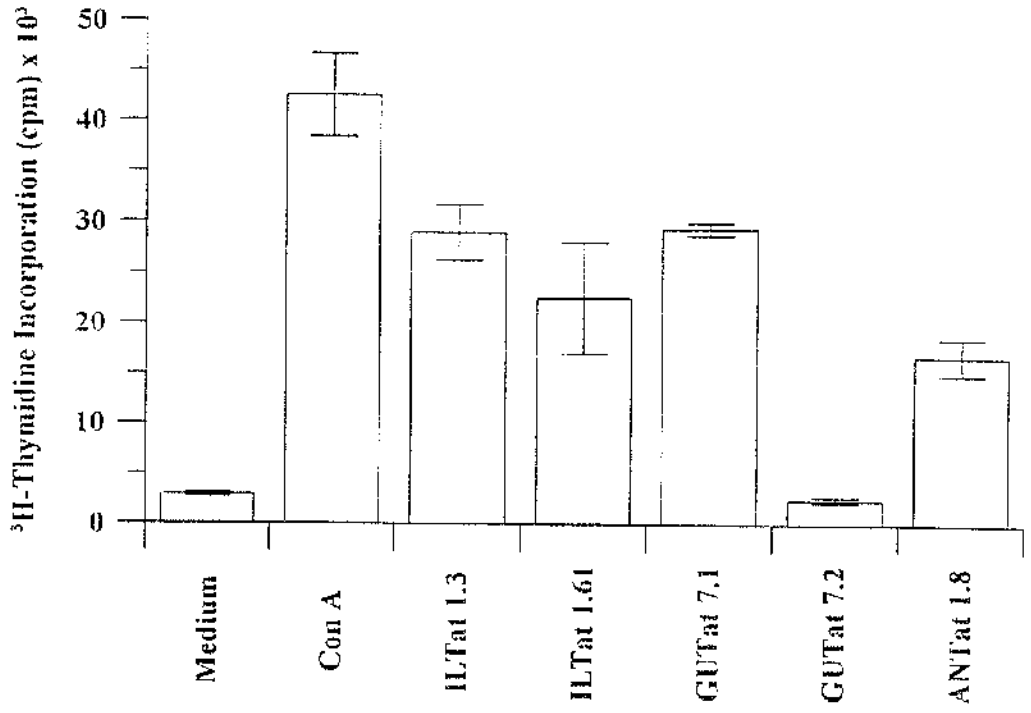

Figure 4.4d. Mononuclear splenocyte proliferation after GUTat 7.2 immunisation.

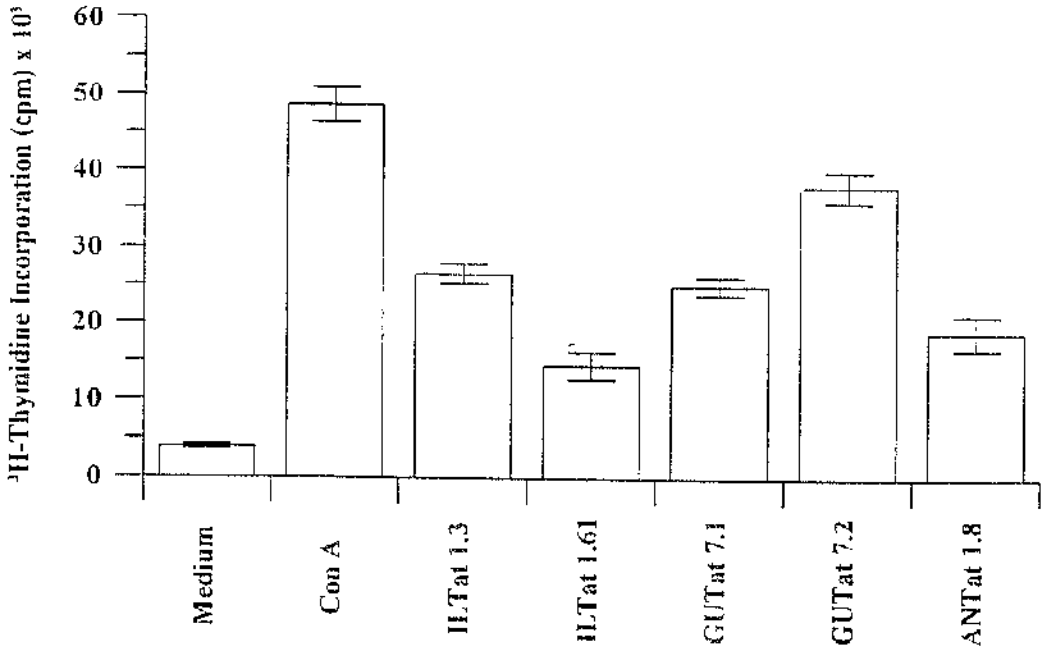

Figure 4.5. Mononuclear splenocytes were harvested from GUTat 7.1-infected mice and analysed by flow cytometry. Cell populations which were incubated with a single antibody were shown as histograms whereas those populations which were double labelled were expressed as dot-plots. The lymphocyte population was gated (**a**) then examined for autofluorescence (**b**). The numbers of B-cells (**c**), T-cells (**d**), CD4 and CD8 cells (**e**), NK cells (**f**), activated CD4 cells (**g**) and activated CD8 cells (**h**) were determined by expressing the positively stained cells as histograms and dot plots.

Figure 4.5. Dot-plots and histograms of cell populations using the mononuclear splenocytes from GUTat 7.1-infected mice.

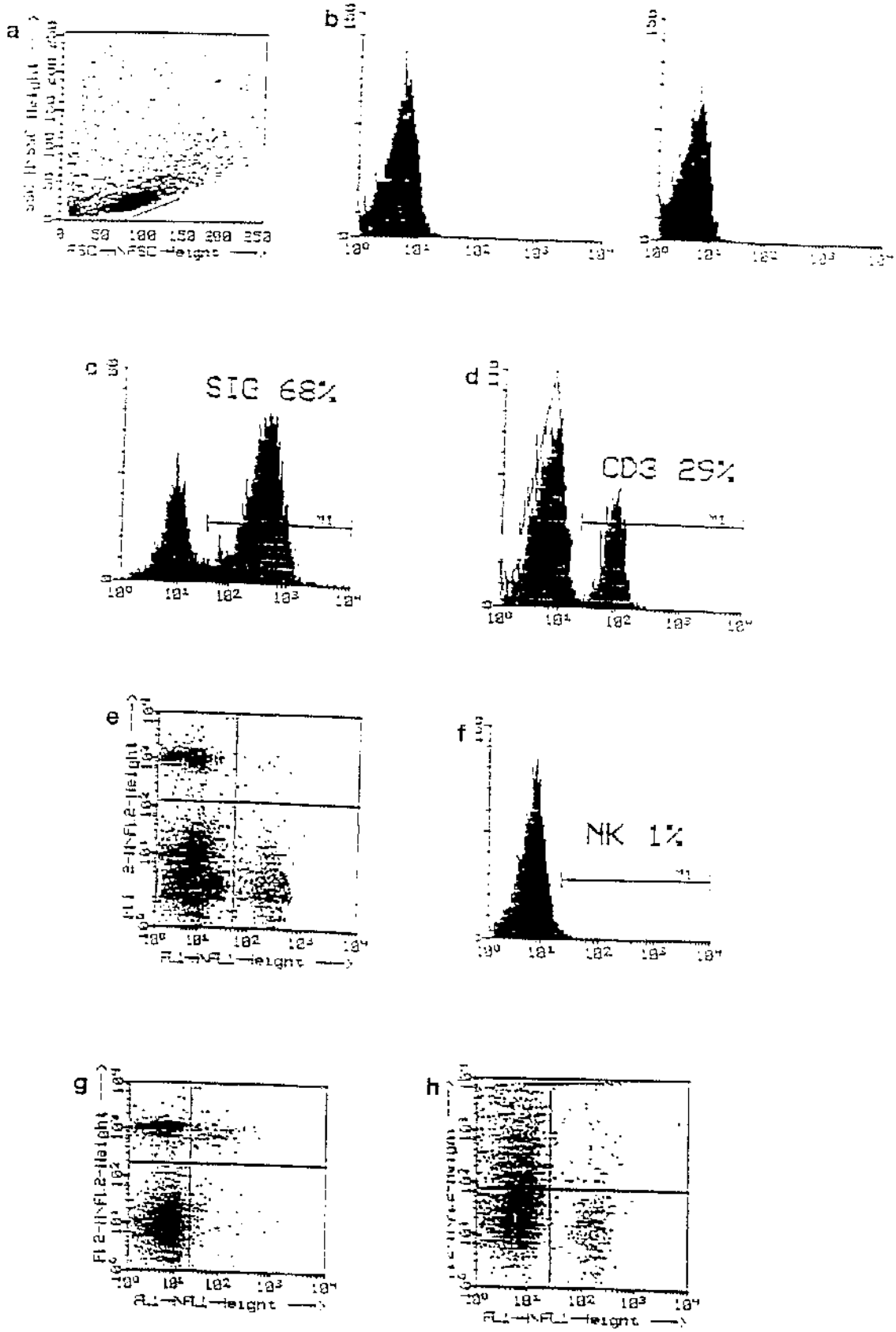

Figure 4.6. The lymphocyte population was gated in the mononuclear splenocyte suspensions from infection and from immunisation with trypanosomes expressing one of four different VATs and compared with mononuclear splenocytes from uninfected control mice. The trypanosome-infected spleens were harvested at the first peaks of parasitaemia for each of the four VATs shown (**a**) and the spleens from the mice immunised with 10^6 paraformaldehyde-fixed trypanosomes were killed two weeks after immunisation (**b**). 5,000 cells were counted for each sample and results are expressed as number of cells per spleen.

Figure 4.6a. Flow cytometry analyses on mononuclear splenocytes from trypanosome-infected mice.

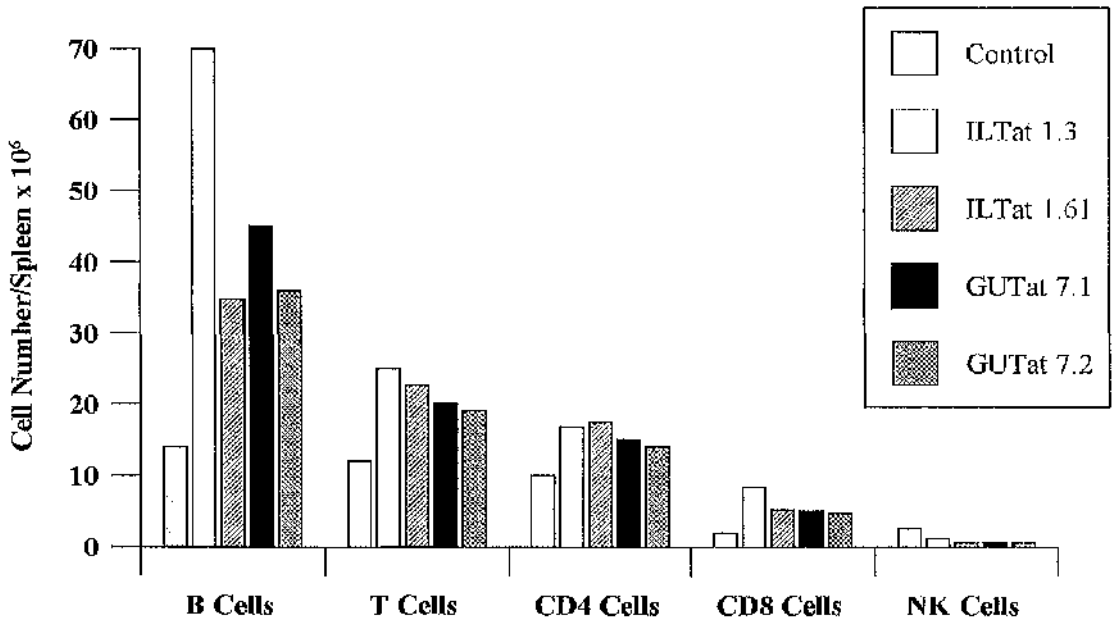

Figure 4.6b. Flow cytometry analyses of mononuclear splenocytes after immunisation.

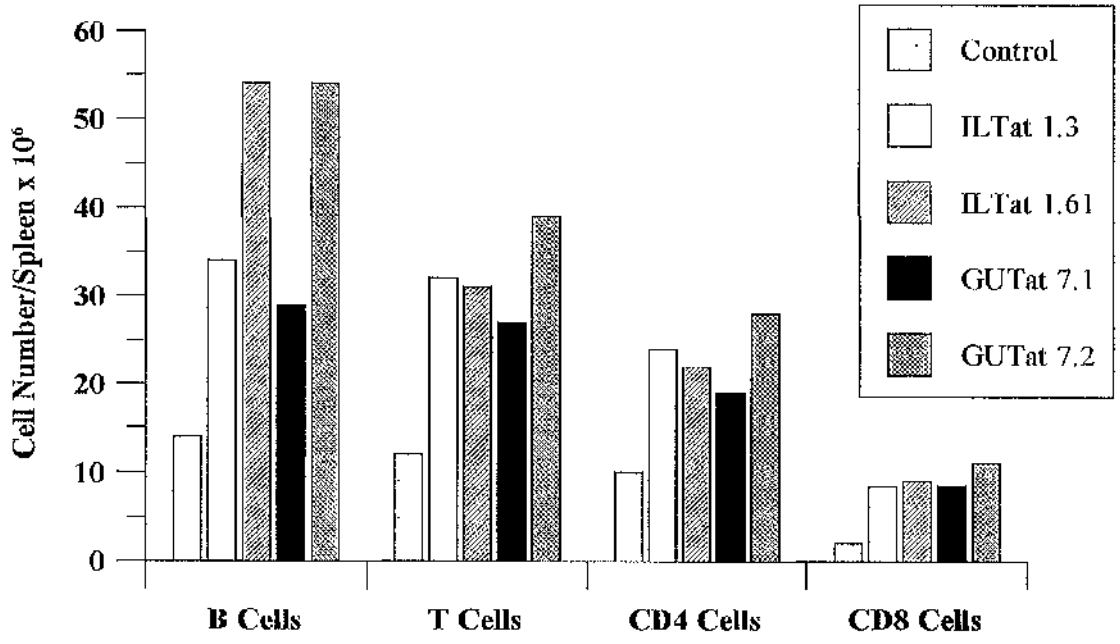

Table 4.4a. Flow cytometry analyses of mononuclear splenocytes from trypanosome infected mice.

Cell Type	Control	ILTat 1.3	ILTat 1.61	GUTat 7.1	GUTat 7.2
B	49	58	60	68	61
T	41	26	39	30	32
CD4	34	18	30	22	24
CD8	7	8	9	8	8
NK	9	1	1	1	1
B:T Ratio	1.2:1	2.3:1	1.5:1	2.3:1	1.9:1
CD4:CD8 Ratio	5:1	2.3:1	2.8:1	2.8:1	2.9:1
CD4:CD25 Ratio	17:1	4.5:1	10:1	7.3:1	8.5:1
CD8:CD25 Ratio	0	8:1	9:1	8:1	9:1

The relative proportions, expressed as percentages. and ratios of splenocytes from mice infected with four different VATs, each at first peak of parasitaemia and mononuclear splenocytes from an uninfected control for comparison.

Table 4.4b. Analyses by flow cytometry of mononuclear splenocytes from immunised mice.

Cell Type	Control	ILTat 1.3	ILTat 1.61	GUTat 7.1	GUTat 7.2
B	49	45	54	45	49
T	41	42	31	42	35
CD4	34	31	22	29	25
CD8	7	11	9	13	10
B:T Ratio	9	1.1:1	1.7:1	1.1:1	1.4:1
CD4:CD8 Ratio	1.2:1	2.9:1	2.4:1	2.2:1	2.5:1
CD4:CD25 Ratio	5:1	33:1	33:1	33:1	33:1
CD4:CD25 Ratio	17:1	49:1	0	50:1	100:1

Mononuclear splenocytes were purified from four groups of mice which were immunised with paraformaldehyde fixed trypanosomes expressing different VATs two weeks after immunisation. The results are expressed as a percentage of the total mononuclear population except where a ratio is stated.

Figure 4.7. Mice were inoculated with one of four trypanosome VATs: ILTat 1.3 (**a**), ILTat 1.61 (**b**), GUTat 7.1 (**c**) and GUTat 7.2 (**d**). At the first peaks of parasitaemia the splenocytes were harvested and cultured *in vitro* in the presence of medium alone, Con A or paraformaldhyde-fixed trypanosomes expressing one of five VATs. IFNγ production was determined by cytokine ELISAs from supernatants harvested at 24 (black) and 72 (white) hours. The results are expressed as the mean of three wells minus the background levels, that is the levels given by the wells which did not contain any of the samples. 1500U/ml was the highest level of IFNγ detected in this assay.

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Figure 4.7a. IFNγ production of mononuclear splenocytes from ILTat 1.3-infected mice.

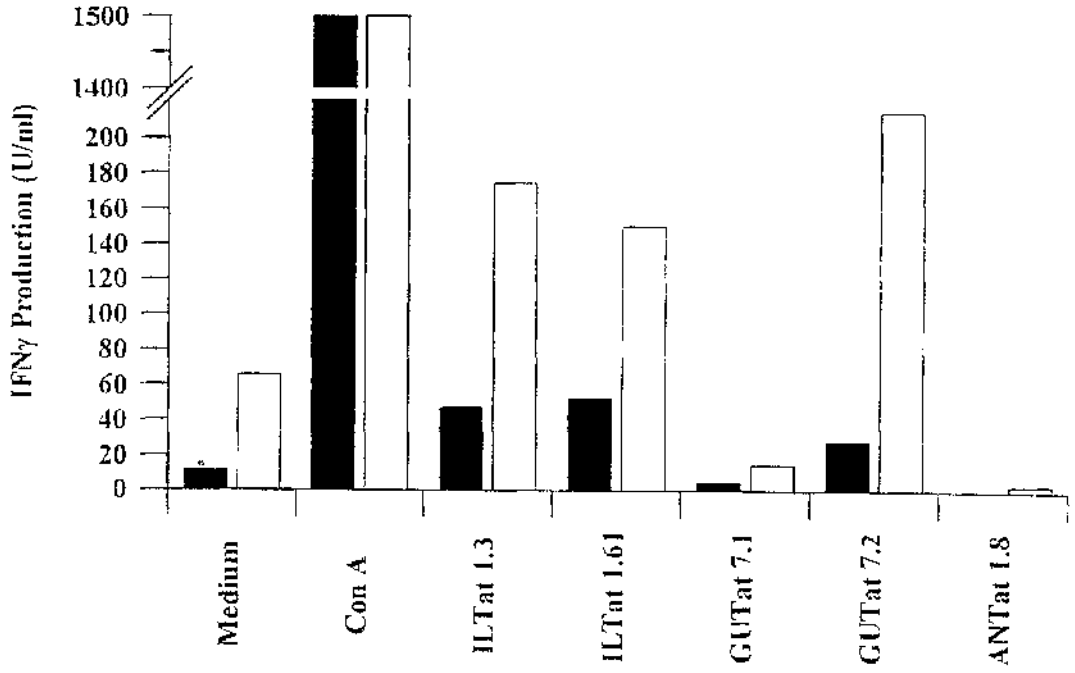

Figure 4.7b. IFNγ production from mononuclear splenocytes of ILTat 1.61-infected mice.

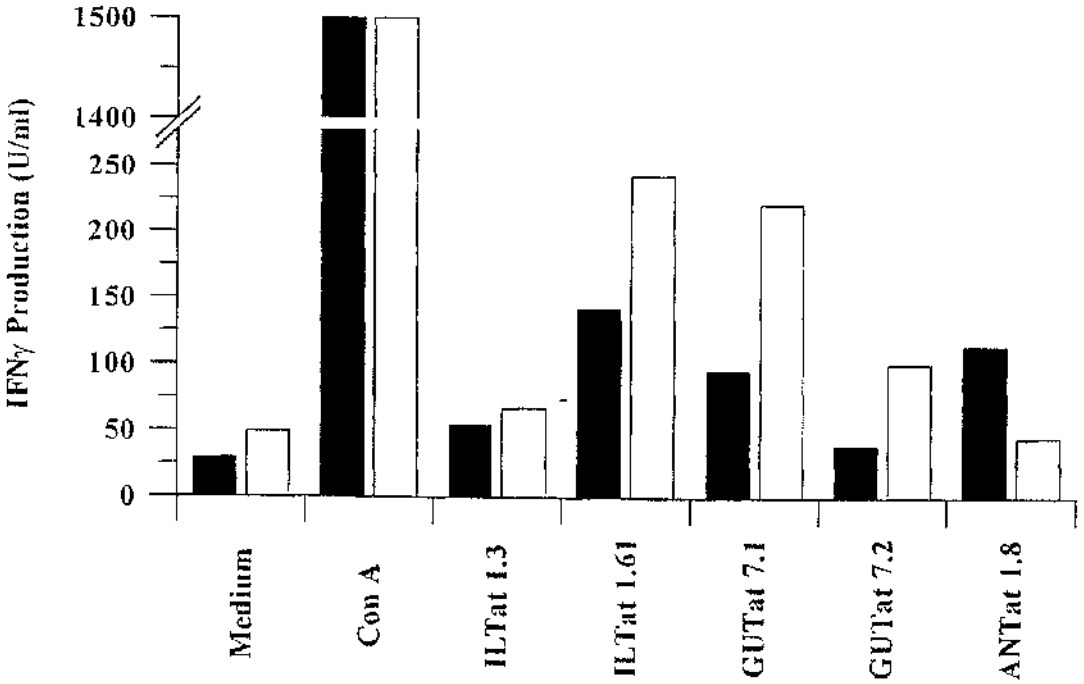

Figure 4.7c. IFNγ production from mononuclear splenocytes of GUTat 7.1-infected mice.

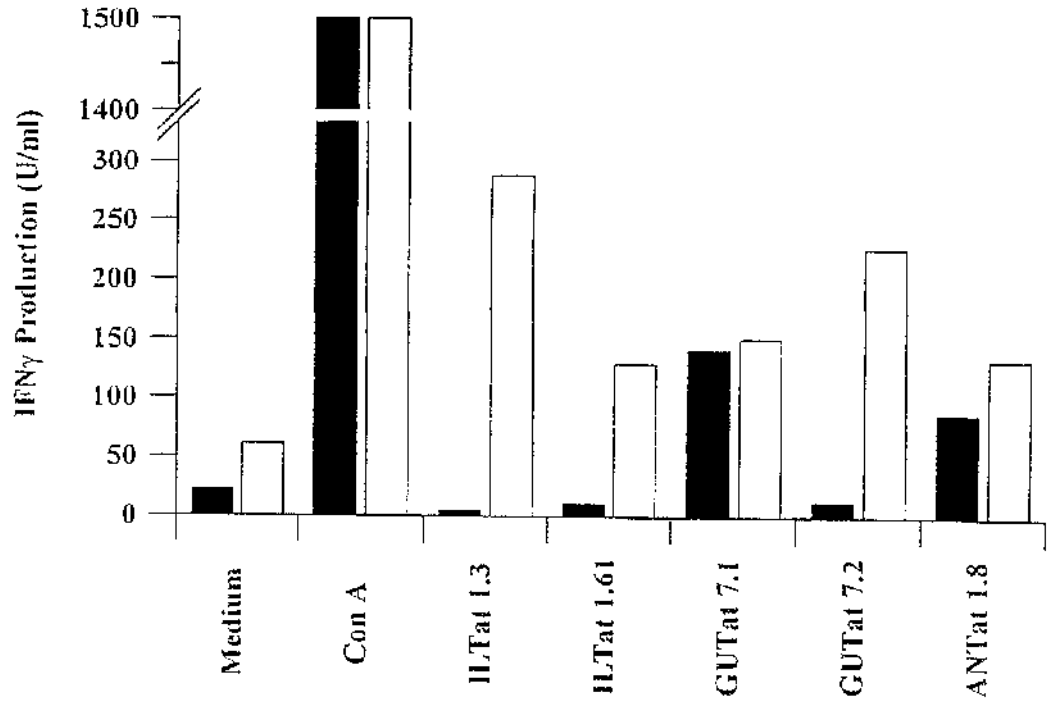

Figure 4.7d. IFNγ production of mononuclear splenocytes from GUTat 7.2-infected mice.

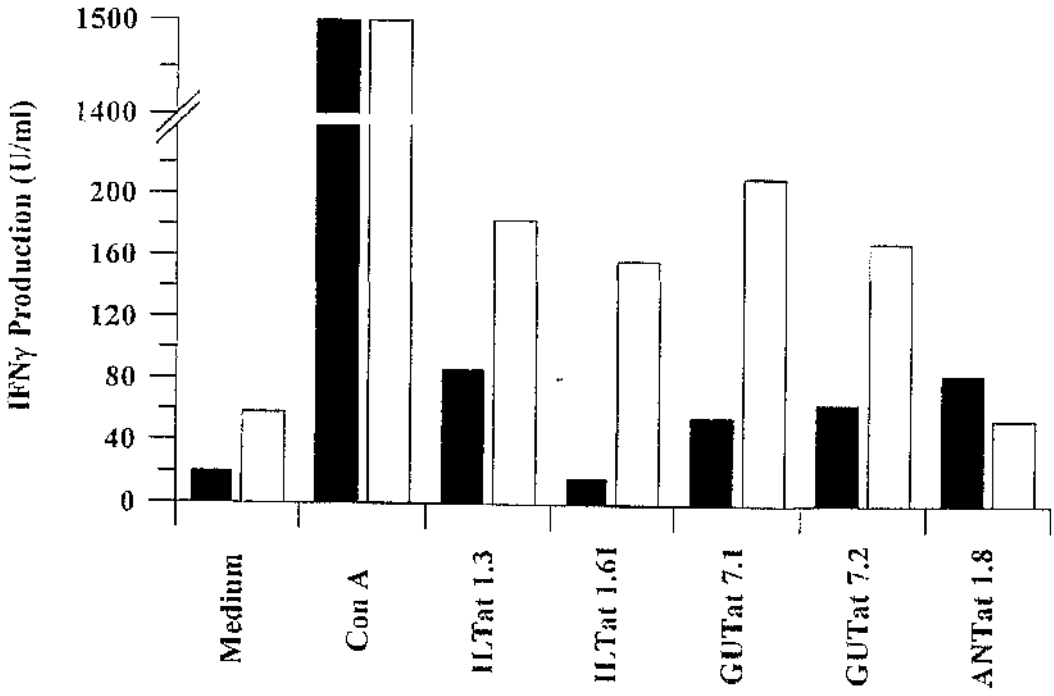

Figure 4.8. Mice were infected with GUTat 7.2 trypanosomes and killed two days after the peak of parasitaemia. The mononuclear splenocyte populations were harvested and cultured *in vitro* in the presence of medium alone, Con A or paraformaldehyde-fixed trypanosomes expressing one of five VATs. IFNγ production was determined from supernatants harvested at 24 (black) and 72 (white) hours. Results are shown as the means of triplicate wells with background levels, that is the reading from the wells which did not contain any sample, subtracted.

Figure 4.9. Mice were immunised with paraformaldehyde-fixed trypanosomes expressing one of four different VATs. The mononuclear splenocyte populations were harvested two weeks later and IFNγ production was determined by cytokine ELISAs. The mononuclear splenocyte populations were stimulated *in vitro* with Con A (8μg/ml) and supernatants were harvested at 24 (black) and 72 (white) hours. The means of triplicate wells are shown minus background levels, that is the levels given by the wells which did not contain any sample.

Figure 4.8. IFNγ production of mononuclear splenocytes from GUTat 7.2-infected mice at the decline of parasitaemia.

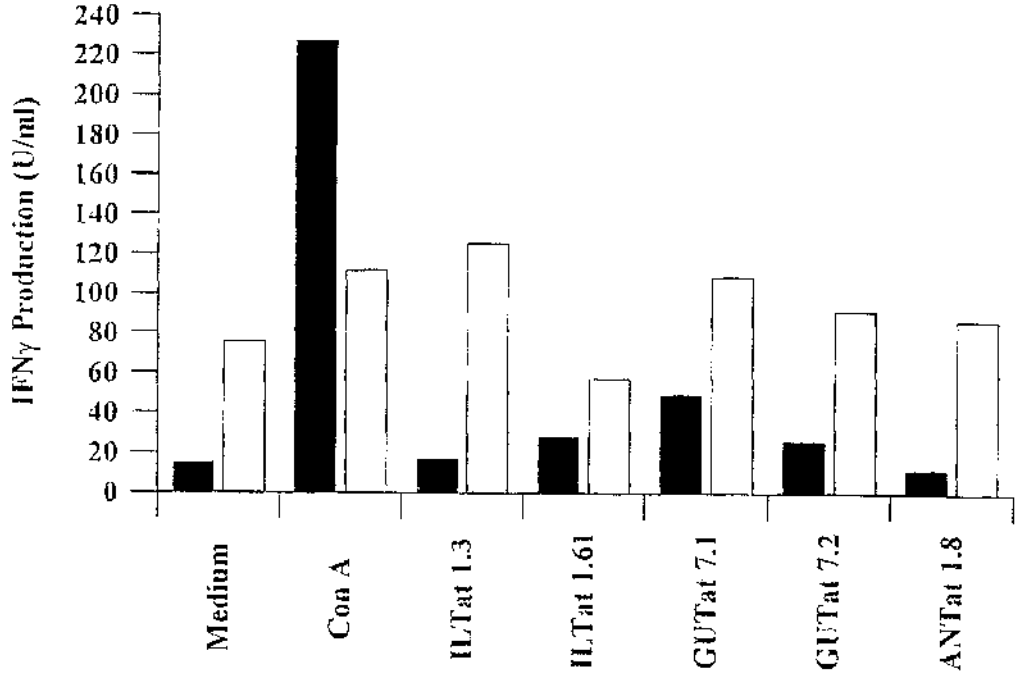

Figure 4.9. IFNγ production from mononuclear splenocytes of immunised mice.

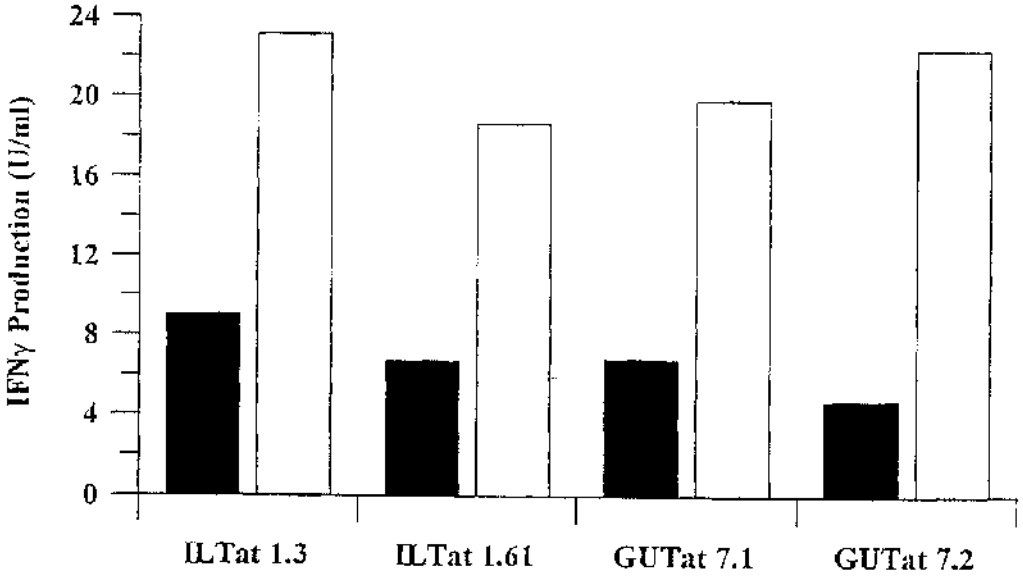

Figure 4.10a. Mononuclear splenocytes from uninfected spleens, infected spleens from the first peak of parasitaemia and from spleens two days after the first peak were harvested. The cells were cultured *in vitro* in the presence of Con A, 8μg/ml, and the supernatants harvested at 24 (black) and 72 (white) hours. Cytokine ELISAs were used to determined IL-2 production from each sample. The results are expressed as the mean of three wells minus the background readings. that is the reading from the wells which did not receive any of the samples.

Figure 4.10b. A time course of IL-2 production by mononuclear splenocytes from mice inoculated with ILTat 1.3, GUTat 7.1 or given a sham injection of PBS. in response to stimulation *in vitro* with Con A (8μg/ml). The first peak of parasitaemia was at 96 hours in both infections. Splenocytes were harvested from the mice at a number of time points and the supernatants were harvested after 24 hours in each case. 500U/ml was the top standard in this assay. The results are expressed as the mean of triplicate wells minus the background readings, that is the levels from the wells which did not contain any of the samples.

Figure 4.10a. Concanavalin A stimulation of IL-2 production from splenocytes from uninfected and infected mice.

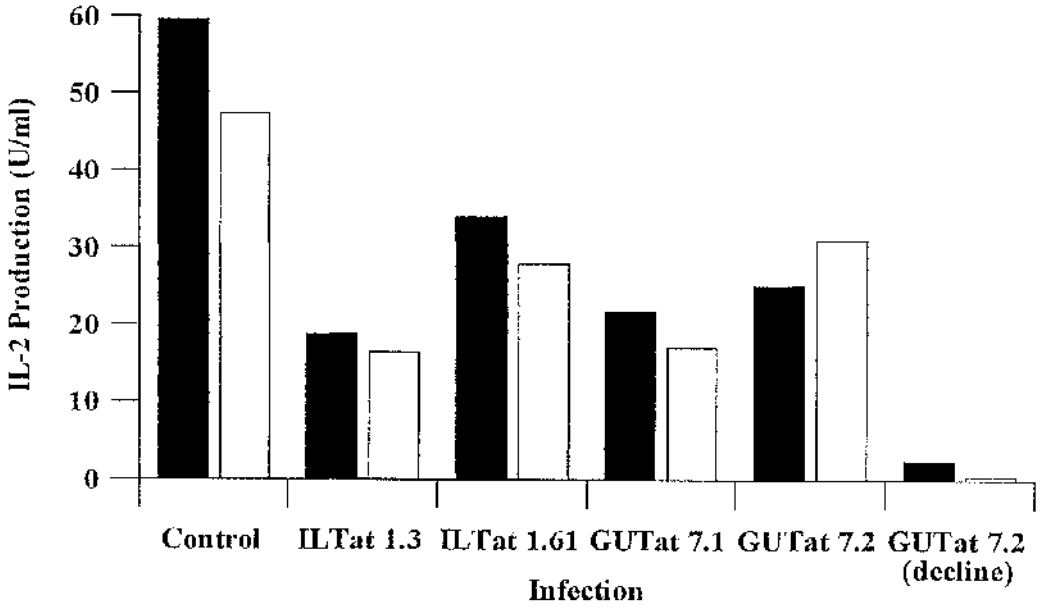

Figure 4.10b. Time course of IL-2 production in the acute-phase of trypanosome infection, before the first peak of parasitaemia.

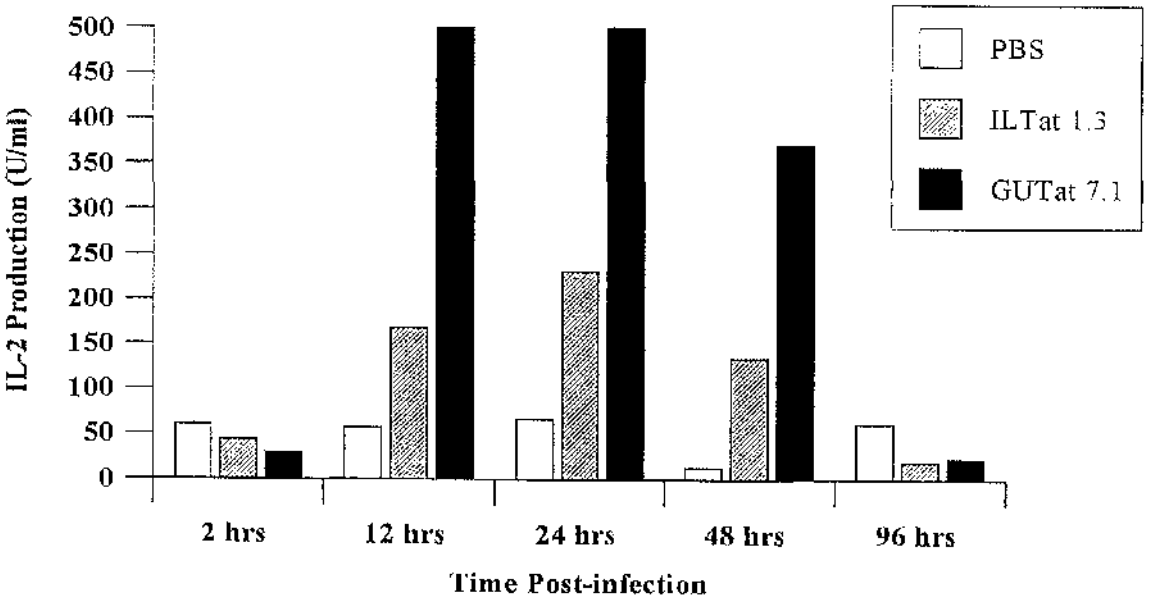

Figure 4.11. Mice were either infected with ILTat 1.3 trypanosomes, drug cured at the peak of parastiaemia and killed one or two weeks later (**a**) or immunised with one of four different paraformaldehyde-fixed trypanosome VATs and killed two weeks later (**b**). The mononuclear splenocyte populations were purified and stimulated *in vitro* with Con A (8μg/ml). The supernatants assayed for IL-2 production at 24 (black) and 72 (white) hours after dispensing. Results are expressed as the mean of triplicate wells with the background levels, that is the readings produced in the wells without the addition of sample, subtracted.

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Figure 4.11a. Production of IL-2 from mice infected, drug cured and splenocytes removed one or two weeks later.

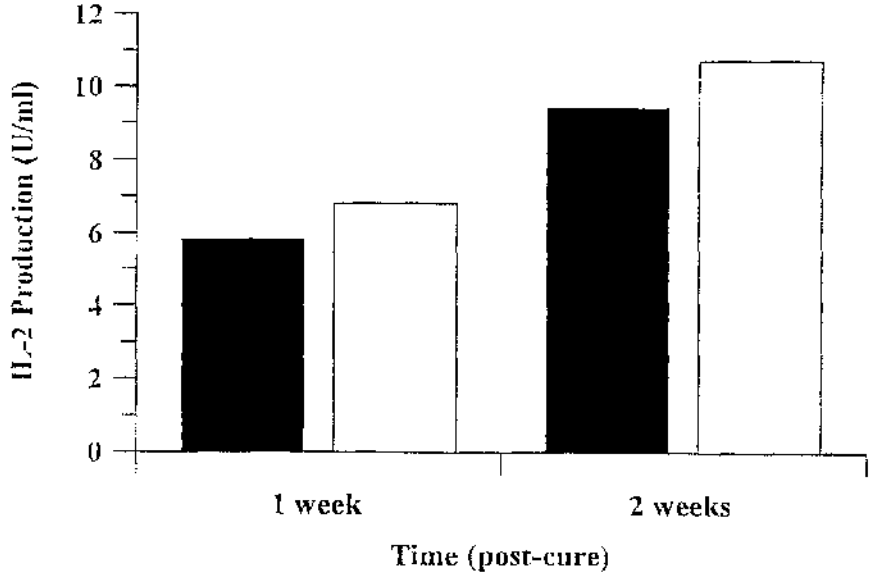
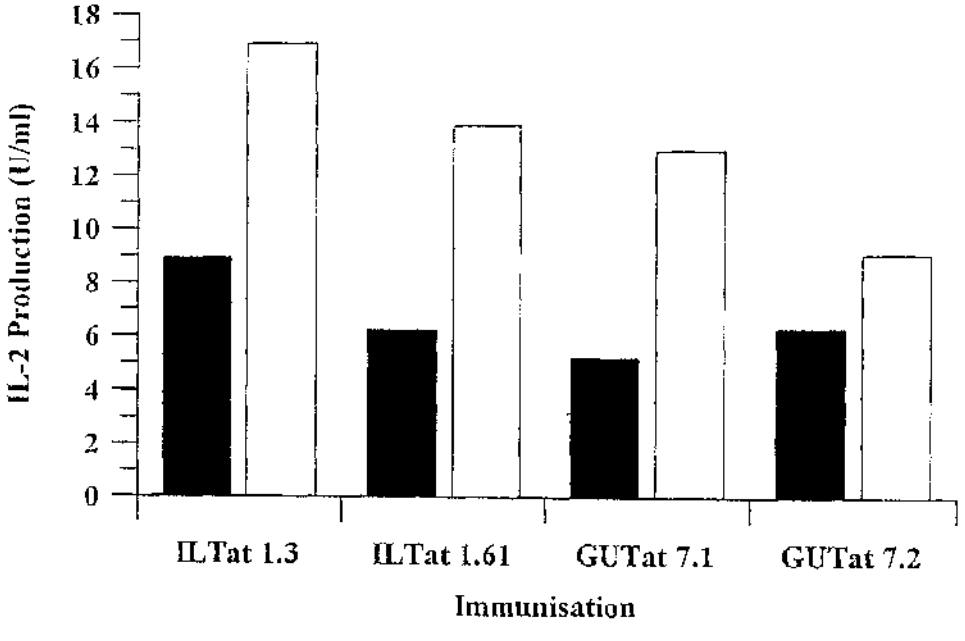


Figure 4.11b. Production of IL-2 from mononuclear splenocytes of immunised mice.



CHAPTER 5

IDENTIFICATION OF POTENTIAL T-CELL EPITOPES ON VARIANT SURFACE GLYCOPROTEINS OF TRYPANOSOMES: A THEORETICAL APPROACH

5.1. INTRODUCTION

The surface coat encapsulates an individual *T.brucei* parasite with the function of protecting the trypanosome from attack by the non-specific immune responses. The coat is highly immunogenic and readily generates adaptive immune responses but by undergoing antigenic variation, the trypanosomes can change their surface coat as a means of evading consequences of immunity (Borst & Cross,1982; Esser & Schoenbechler,1985; Turner & Barry,1989; Borst,1991). The surface coat consists of a monolayer of a single molecular species in high copy number, $>10^7$ copies per cell; the VSG. Antigenic variation is enacted by switching from expression of one VSG to that of another. Although there are several hundred VSGs that can be expressed by each cell they all, as far as has been analysed to date, have several conserved features that they show. VSGs consist of a carboxy terminus, which is relatively constant in its amino acid sequence and contributes to one third of the VSG, the remaining two thirds being the highly variable amino terminus, which contains large hypervariable regions. The carboxyl terminal is linked to the parasite membrane via a glycophosphatidyl inositol (GPI) anchor. The primary sequences of the VSGs differ substantially but the VSG homodimers have highly conserved tertiary structures and two of these tertiary structures

have been determined by x-ray crystallography (Blum *et al*,1993). These structures consist of internal α-helices, and extensive inter- and intramolecular disulphide linkages which provide a rigid column-shaped structure on the plasma membrane (Freymann *et al*,1990). The VSG molecules are tightly packed on the surface membrane of the trypanosome such that large molecules, such as immunoglobulin and C5b, are physically prevented from gaining access to the membrane itself. On live parasites antibodies only recognise a small portion of the VSG molecule in the amino terminal domain, distal to the plasma membrane (Magnus *et al*,1982; Sendashonga & Black,1982; Masake *et al*,1983; Black,1986). There is no reasonable explanation as yet as to why hypervariability in amino acid sequence should be found not only in these regions of the molecule, which are subject to effector antibody responses, but also in most other (subsurface) areas of the amino terminal domain. One possible explanation is to evade T-cell responses.

Antibodies can distinguish between tertiary protein structures extremely well unlike T-cells which only recognise processed antigen and cannot therefore discriminate between native and denatured proteins. The T-cells can therefore come into contact with the hypervariable regions buried within the tertiary VSG structure when the VSG is processed and cleaved into peptides. Antibodies capable of effective killing of live parasites can only be generated against exposed regions of the intact tertiary structure. Once a parasite has been killed, antibodies can be generated against subsurface regions of VSGs and internal invariant antigens but these antibodies will be ineffective in killing trypanosomes (Pearson *et al*,1986; Shapiro,1994).

The experimental literature on T-cell responses in trypanosome infections is confused. Athymic nu/nu mice can sufficiently control trypanosome infections by IgM and immune lysis (Campbell *et al*,1978). Irradiated thymectomised adult mice survive *T.brucei* better

than fully immunocompetent mice (Askonas *et al*,1979). Together, these studies suggest that the antibody response is T-independent. In contrast, irradiation and reconstitution of chimeric mice (DeGee & Mansfield,1984) and lack of correlation of longevity of infected mice with quality of antibody response (Sendashonga & Black,1988) both suggested the involvement of T-cells in control of infection. The later direct observation of VSG-specific T-cells in the peritoneal T-cell population of infected mice confirmed the involvement of T-cells (Schleifer *et al*,1993). These VSG-specific T-cells produced cytokines of the T-helper 1 subset but did not proliferate in response to the VSG.

To conduct a prelimary study as to whether VSGs might drive T cell responses and to investigate whether these responses were likely to be restricted to particular VSGs, I analysed VSG sequences using computer algorithms predictive of T cell epitopes. A computer program package, "TSites", was used. This program (Feller and De La Cruz,1991) uses four independent algorithms to identify potential antigenic determinants for T-cells on amino acid sequences. The basis for each of these four algorithms is as follows:

1.) An amphipathic structure is one which has hydrophilic and hydrophobic surfaces at opposite faces and the amphipathic helix model (Margalit *et al*,1987) is based on the theory by Delisi and Berzofsky (1985) that T-cell epitopes are predominantly amphipathic α-helices. This algorithm was shown by the original authors to be 75% successful and was highly significant ($p<0.001$) in identifying a battery of known T cell epitopes. This algorithm has identified known helper and cytotoxic T-cell epitopes in other studies (Margalit *et al*,1987; Reyes *et al*,1990; Vordermeier *et al*,1993).

2.) The Rothbard motif (Rothbard and Taylor,1988) is based on findings that either one of two possible amino acid sequences are identified and recognised by MHC Class I and

II for presentation. This motif has also been used successfully to identify $CD4^+$ and $CD8^+$ T-cell epitopes (Reyes *et al*,1990; Ashbridge *et al*,1992 Vordermeier *et al*,1993). 3.) & 4.) Sette *et al* (1989) showed a 75% success rate in identifying T-cell epitopes with each of the $I\text{-}A^d$ and $I\text{-}E^d$ algorithms. These motifs are based on the likelihood of a segment of protein binding to the $I\text{-}A^d$ or the $I\text{-}E^d$ molecule, respectively, of the murine MHC H-2 haplotype. Known T-cell epitopes were used with all of these algorithms as a comparison in the method of identifying T-cell epitopes with regards to efficiency and sensitivity (Reyes *et al*,1990).

These T-cell prediction algorithms have been employed in the present study to investigate which regions of VSGs are potential T-cell epitopes and to determine whether any of these epitopes are relatively conserved between the primary sequences if different VSGs or if they vary considerably between molecules. Because the tertiary structures of the VSGs are highly conserved (Blum *et al*,1993) and other information suggests that such conservation extends to many, if not all, VSGs (Blum *et al*,1993), including some of those used in experimental studies in the previous chapter, it has been possible to suggest how predicted T-cell epitopes map onto the tertiary structure of the VSG molecule.

5.2. RESULTS

T-cell epitopes predicted by "TSites" were identified in five VSG sequences. These five sequences were chosen for analysis because for two of them (ILTat 1.24 and MITat 1.2) the tertiary structures have been resolved and the other three VSGs (ILTat 1.3, ILTat 1.61 and ILTat 1.22) were used in experimental studies in Chapter 4. The

sequences of four of the VSGs were published before the start of the project (Rice-Ficht *et al*,1981; Carrington *et al*,1991) and ILTat 1.61 was kindly sequenced by Dr. Mark Carrington, University of Cambridge at our request. The sequence of this new VSG is given in Figure 5.1e. ILTat 1.61 is an atypical Class A VSG with respect to its amino domain sequence and is such it is extremely difficult to align its sequence to that of other VSGs (Carrington & Turner, unpublished results).

For all the sequences analysed, "TSites" predicted a number of potential epitopes widely distributed throughout the sequence [Figure 5.1a-e.]. No algorithm is 100% accurate in its predictions but use of four algorithms in combination should have increased the confidence with which predictions can be made. A few epitopes are highlighted as particular strong candidate epitopes.

Guidelines were adopted when selecting these epitopes so as to only select the most likely candidate T-cell epitopes within the sequences. The AMPHI motif was never used on its own as an indicator of T-cell epitopes because the VSG tertiary structures consist of two large amphipathic helices running vertically through the molecule which may be identified instead of potential T-cell epitopes. Another reason for not solely relying on this motif is that if a region is identified as amphipathic then it will not necessarily be antigenic but the converse situation may well apply: if the identified region is antigenic then there is a high probability that it will be amphipathic (Delisi & Berzofsky,1985). Furthermore, I decided that at least two motifs must recognise the potential epitopes before the epitope is selected. This does not necessarily mean that those regions identified by only one motif are not likely T-cell epitopes, only that there is an increased probability of successful identification if there are two or more motifs recognising a particular region of the amino acid sequence.

A minumum of four amino acids, which are recognised by two or more motifs as part of a potential epitope, are highlighted. The potential CD4 epitopes are shown in bold whereas the potential CD4/8 epitopes are in bold and underlined. The residues surrounding these highlighted amino acids will contribute to the remainder of the epitope. If the key residues for the MHC binding groove or the T-cell receptor are within these four amino acids then they may form part of a T-cell epitope. Many other potential T-cell epitopes have been identified on these primary sequence but have not been highlighted.

The alignment of these sequences shows interesting comparative distributions of epitopes [Figure 5.2.]. The variability in distribution of identified epitopes between sequences is dramatic and perhaps best illustrated by focussing on regions of the sequence that define conserved features of the tertiary structure. Such features are, for example, Cysteine 15 or Glycine 247, as defined by Blum *et al* (1993).

To address the question as to whether some of these epitopes are exposed on the surface of the VSG molecule on intact cells, where they are potentially available to antibodies, or buried within the VSG coat, where any variation between epitopes can definitively not be attributed to evasion of functionally important effector antibody responses, I mapped some epitopes onto the resolved 3-D tertiary structures [Figure 5.3.]. The predicted best candidate epitopes for the MITat 1.2, ILTat 1.3, ILTat 1.61 and ILTat 1.22 sequences were used, mapped onto the structure of MITat 1.2. Analysis of this figure reveals that although some epitopes are located on the surface area of the VSG there are a number of epitopes which are subsurface, that is within the VSG coat, which could not come into contact with antibodies. These data suggest that some epitopes are T-cell epitopes and that variation in the VSG sequences maybe driven (in an evolutionary sense) by evasion of T-cell, rather than antibody, responses.

5.3. DISCUSSION

Potential T-cell epitopes have been identified on the amino acid sequences of MITat 1.2, ILTat 1.24, ILTat 1.3, ILTat 1.61 and ILTat 1.22. This last antigen type is an isoVAT of GUTat 7.1 and the two have identical amino acid sequence (Carrington & Turner, unpublished result). MITat 1.2 and ILTat 1.24 were used in this study because their tertiary structures have already been determined (Metcalf *et al*,1987; Freymann *et al*,1990) and the other three are routinely used in experimental studies in the lab. Regions recognised by more than one motif were selected as potential candidate epitopes. Because the VSG tertiary structures have been shown to be highly conserved between antigen types (Blum *et al*,1993) by x-ray crystallography studies, the possible epitopes were mapped onto the 3-D tertiary structure of MITat 1.2. Some identified epitopes are exposed on the tertiary structure to the host immune response but some are subsurface and on live trypanosomes will not come into contact with protective host antibody responses. It may therefore be possible that these regions are subsurface and extremely variable between VSGs to evade T-cell responses in the hostile immune environment. However, there are also some identified epitopes which are similar in locality between the tertiary VSG structures of the different antigen types investigated.

Individually, each of the four algorithms has a very good success rate in identifying T-cell epitopes (Margalit *et al*,1987; Reyes *et al*,1990; Rothbard & Taylor,1988; Sette *et al*,1989) but these algorithms in combination with one another should increase this success rate further. Therefore a region within the primary VSG sequence recognised by one algorithm has a good probability of being a T-cell epitope but if more than one of these algorithms recognises the same region then there should be a very high probability indeed that the region will be identified as a T-cell epitope.

The AMPHI motif is not a good indicator, on its own at least, of potential T-cell epitopes of VSGs. This is due to the fact that there are two very large amphipathic helices through the centre of each VSG and these are likely to be primarily of structural rather than antigenic significance. The recognised regions however could be possible T-cell epitopes but it would not be conclusive unless other motifs were used in conjunction with the AMPHI motif or unless experimental studies were conducted. The regions of the VSG recognised by the Rothbard motif are good indicators of possible CD4 and CD8 T-cell epitopes. The regions recognised by either I-A^d or I-E^d motif are most likely to be MHC Class II regions and therefore potential CD4 T-cell epitopes. The greatest probability for a potential T-cell epitope is a region of the VSG which is recognised by the AMPHI, Rothbard and I-A^d motifs all at one site on the VSG or recognised by the AMPHI, Rothbard and I-E^d motifs at one site.

The possible T-cell epitopes identified on the amino acid sequence of the VSG have been mapped to the tertiary VSG structures to identify those epitopes that are likely to be exposed to the host immune system on the surface of the tertiary structure and those epitopes which are subsurface in the tertiary structure and could be recognised only by T-cells when the VSG is processed and presented by APCs or by antibodies that would be incapable of generating a functional effector response.

The conformation of the antigenic peptides has not been considered because linear determinants are recognised by both helper and cytotoxic T-cells and if the sequences have corresponding features then these will be obvious in the primary protein structure (Rothbard & Taylor, 1988). It is important to note that none of the four algorithms takes into consideration additional structures of peptides that are required for presentation to T-cells (Reyes *et al*, 1990). To be antigenic, peptides must have an anchor, or anchors, depending on whether the peptide groove is of the Class I or II MHC, within the

sequence to allow the peptide to fit correctly into the peptide groove for presentation. It must also have some residues orientated towards the T-cell receptor to allow recognition of the peptide. However, these algorithms have a high success rate in identifying known T-cell epitopes without considering detailed positional information of residues contained within peptides. The potential importance of such information is well illustrated by a study of a CD4 T-cell determinant of gp160 on the human immunodeficiency virus (HIV) (Boehncke *et al*,1993). By substituting different amino acids into different positions within peptides these workers obtained evidence that in Class I MHC molecules there are a very limited number of key peptide residues that are essential for effective binding to allele-specific class I products. Their data also showed that to maintain an epitopic structure which induces a T-cell response is the responsibility of only a few specific amino acids.

Ashbridge *et al* (1992) have successfully used the AMPHI motif, Rothbard motif and the I-A^d motif to identify peptides on a 19kDa protein from *Mycobacterium tuberculosis*. These peptides were synthesised and were found to induce T-cell proliferation *in vitro*. Other studies have been less successful in using T-cell prediction algorithms however. Synthetic peptides were made from tetanus toxin epitopes (Panina-Bordignon *et al*,1989) and although some generated a T-cell response others did not.

If synthetic peptides are produced from these potential T-cell epitope regions recognised on the VSGs, they would not be predicted to all result in the same degree of T-cell response. An illustration of this point comes from a study by Vordermeier *et al* (1993) who found that of 14 *M.tuberculosis* peptides, 12 were immunogenic but to differing degrees. The T-cell proliferation of lymph node and spleen cells were examined and some of the peptides resulted in a very strong T-cell response whereas others induced a weak response.

Despite the flaws in experimental design, the data and results created by the "TSites" program do show several potential T-cell determinants on the VSG amino acid sequences analysed. Due to the high degree of polymorphism that exists within MHC molecules it is not possible to generate peptides which will bind to all alleles although they may bind to several alleles. This however gives a basis for further *in vitro* and *in vivo* investigation into the role or involvement of T-cells in African trypanosome infections and suggests that if several of the most common epitope regions between several VSGs were synthesised into peptides then this may be the basis of a potential vaccine, or alternatively that T-cell responses, as well as antibodies, are effective in driving of antigenic variation.

Figure 5.1. Primary VSG sequence analyses to determine potential T-cell epitopes were carried out using 4 different T-cell prediction algorithms: the 'AMPHI' motif (A); the 'Rothbard & Taylor' motif (R); the 'I-A^d' motif (D) and the 'I-E^d' motif (d). The sequences examined were ILTat 1.24 (**a**), MITat 1.2 (**b**), ILTat 1.3 (**c**), ILTat 1.22 (**d**) and ILTat 1.61 (**e**). The four algorithms make rather different predictions but areas where there is overlap between two or more algorithms are considered reasonable candidate epitopes. These potential CD4 epitopes are highlighted in bold whereas the potential CD4/8 epitopes are highlighted in bold and underlined.

Figure 5.1a. The primary sequence of ILTat 1.24 with predicted CD4 and CD8 epitopes T-cell identified.

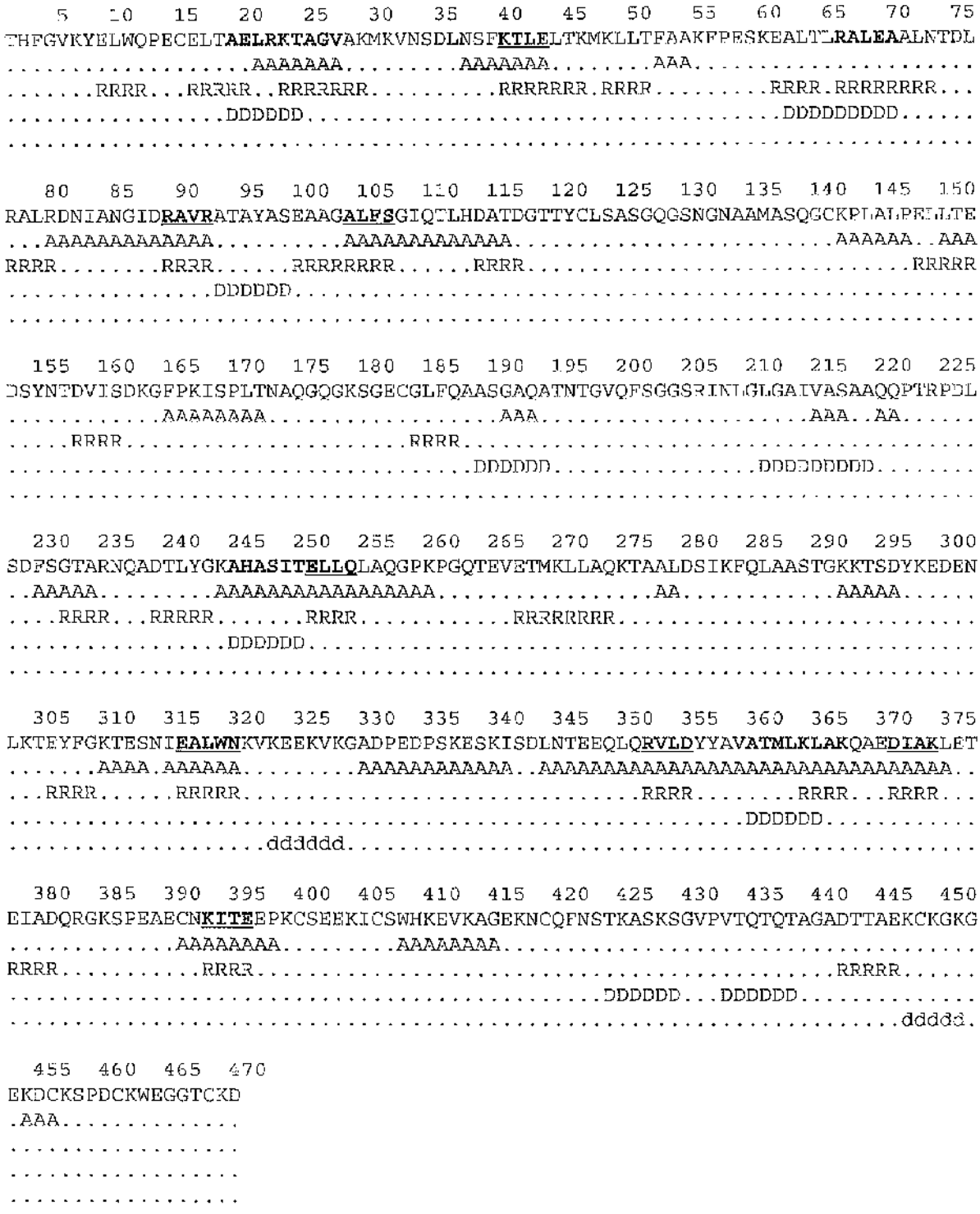


Figure 5.1b. The amino acid sequence of MITat 1.2 with the predicted T-cell epitopes identified.

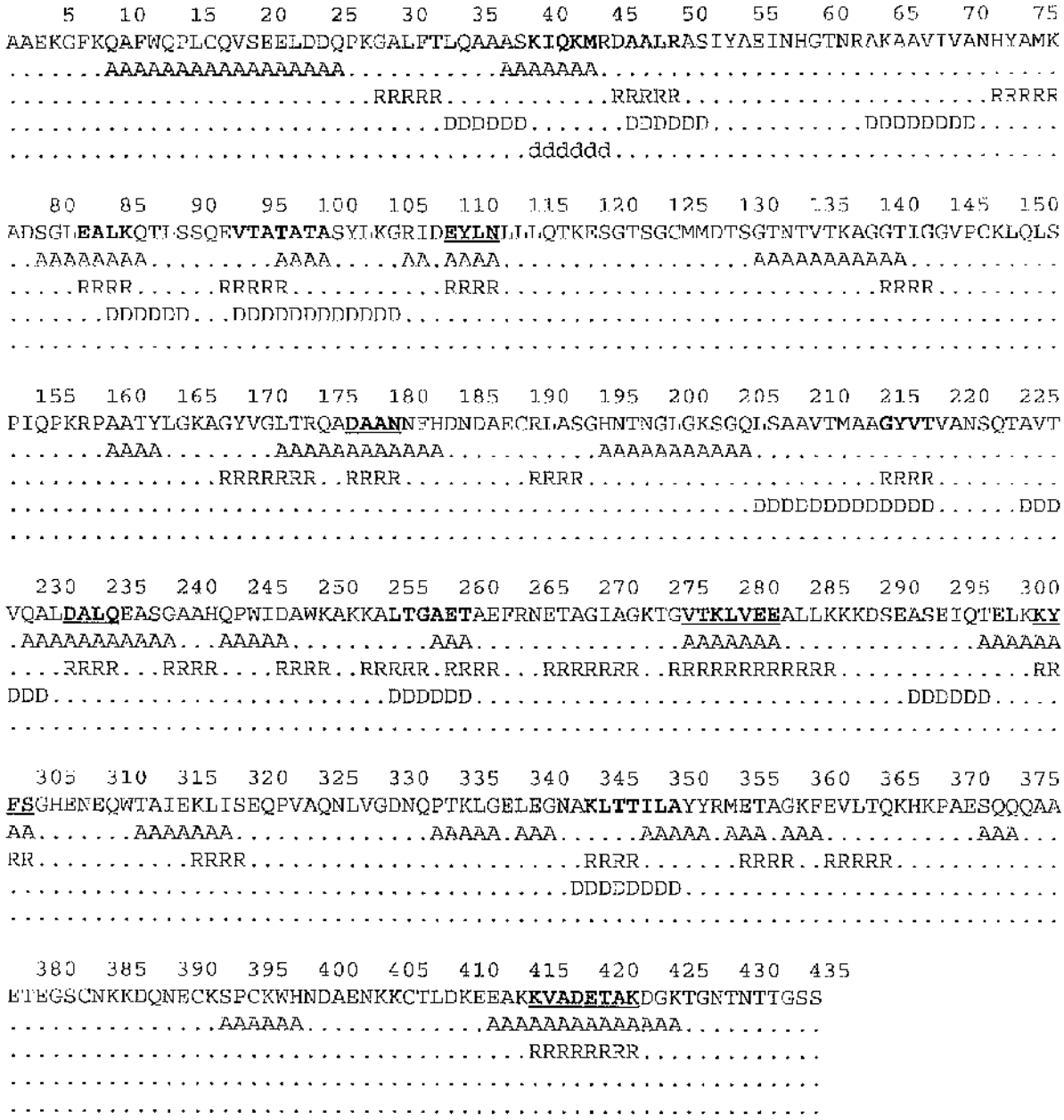


Figure 5.1c. HTat 1.3 amino acid sequence with predicted CD4 and CD8 epitopes identified.

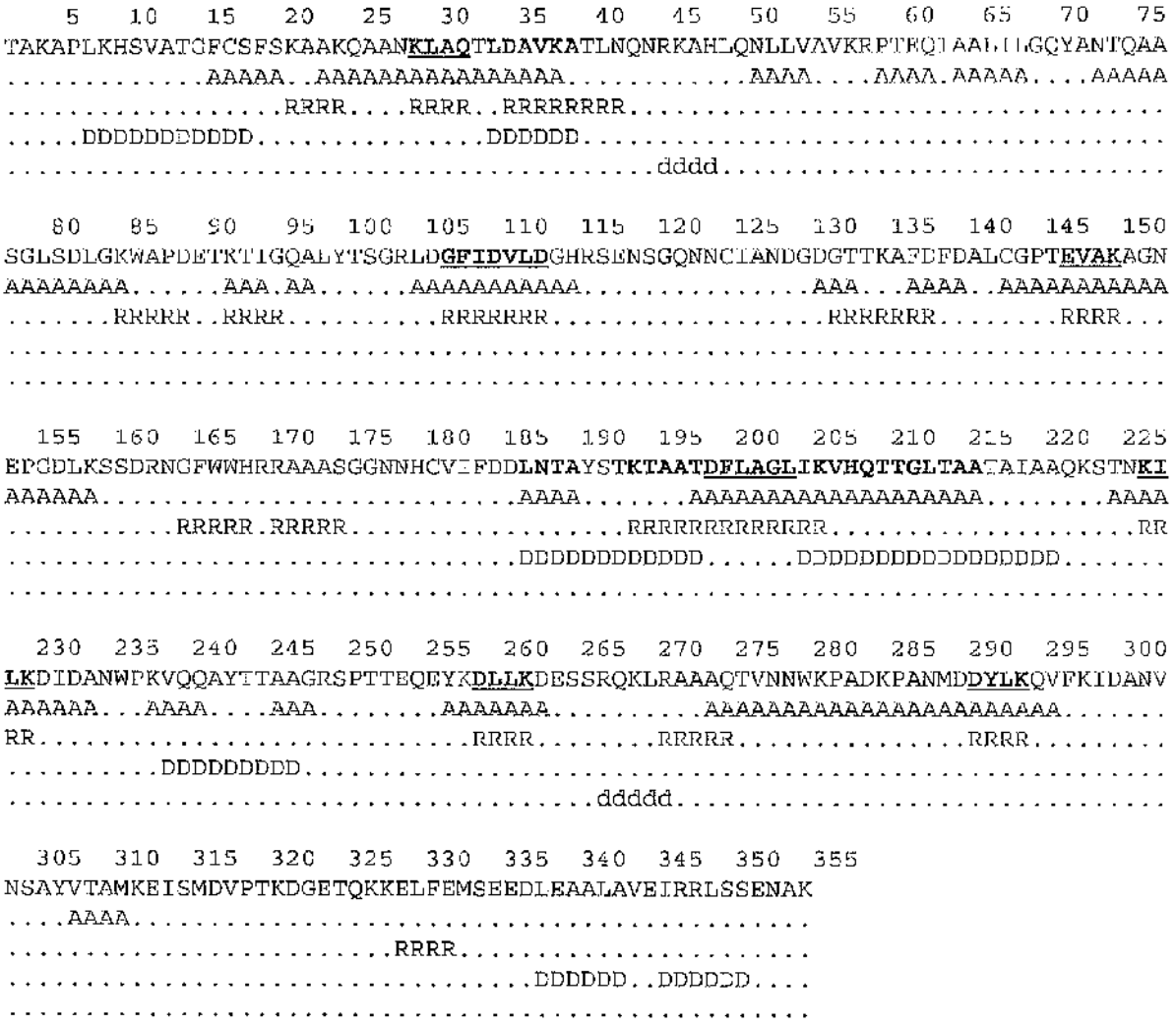


Figure 5.1d. Identified potential T-cell epitopes on the primary sequence of ILTat 1.22.

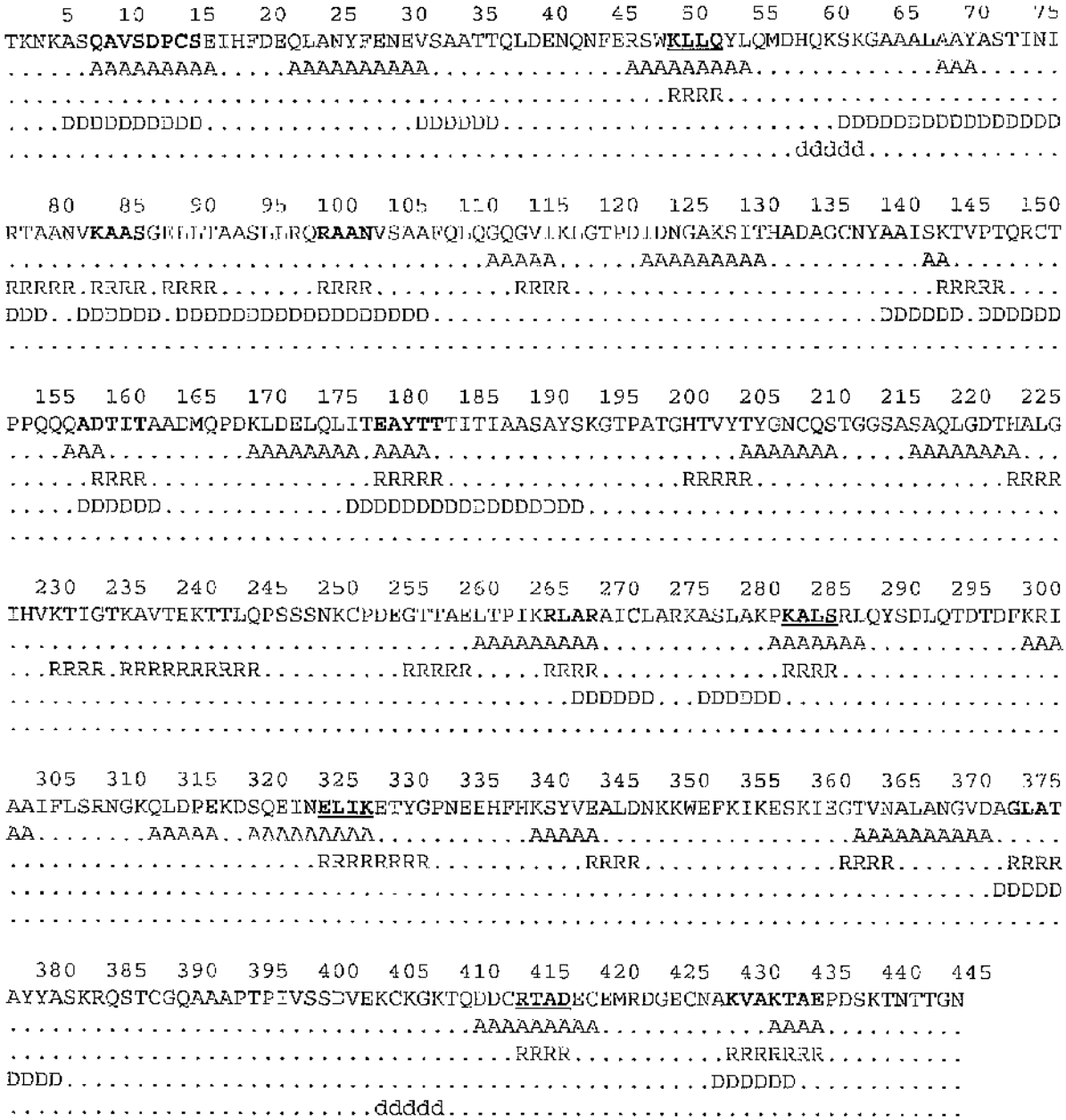


Figure 5.1c. Algorithms identifying predicted T-cell epitopes on the ILTat 1.61 amino acid sequence.

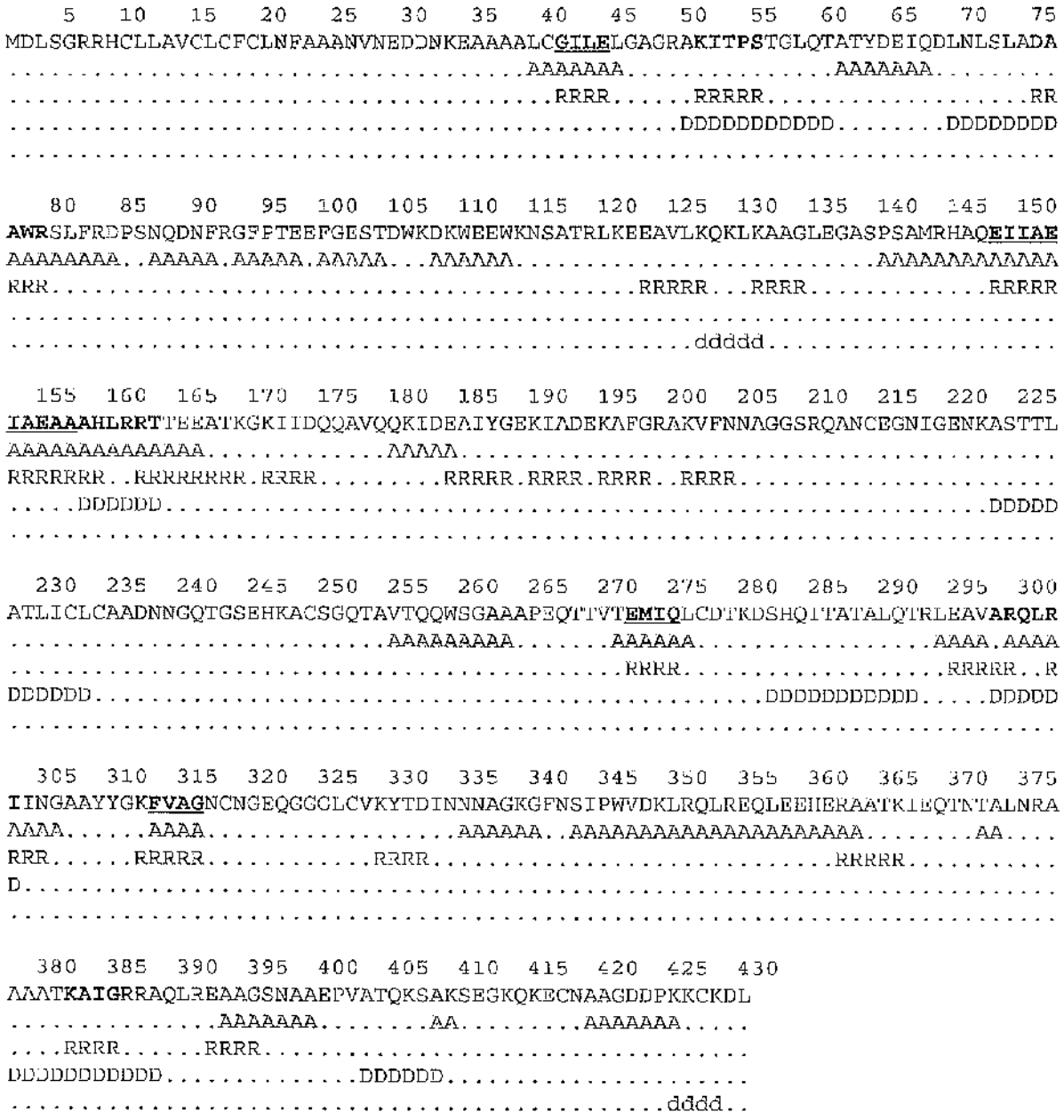
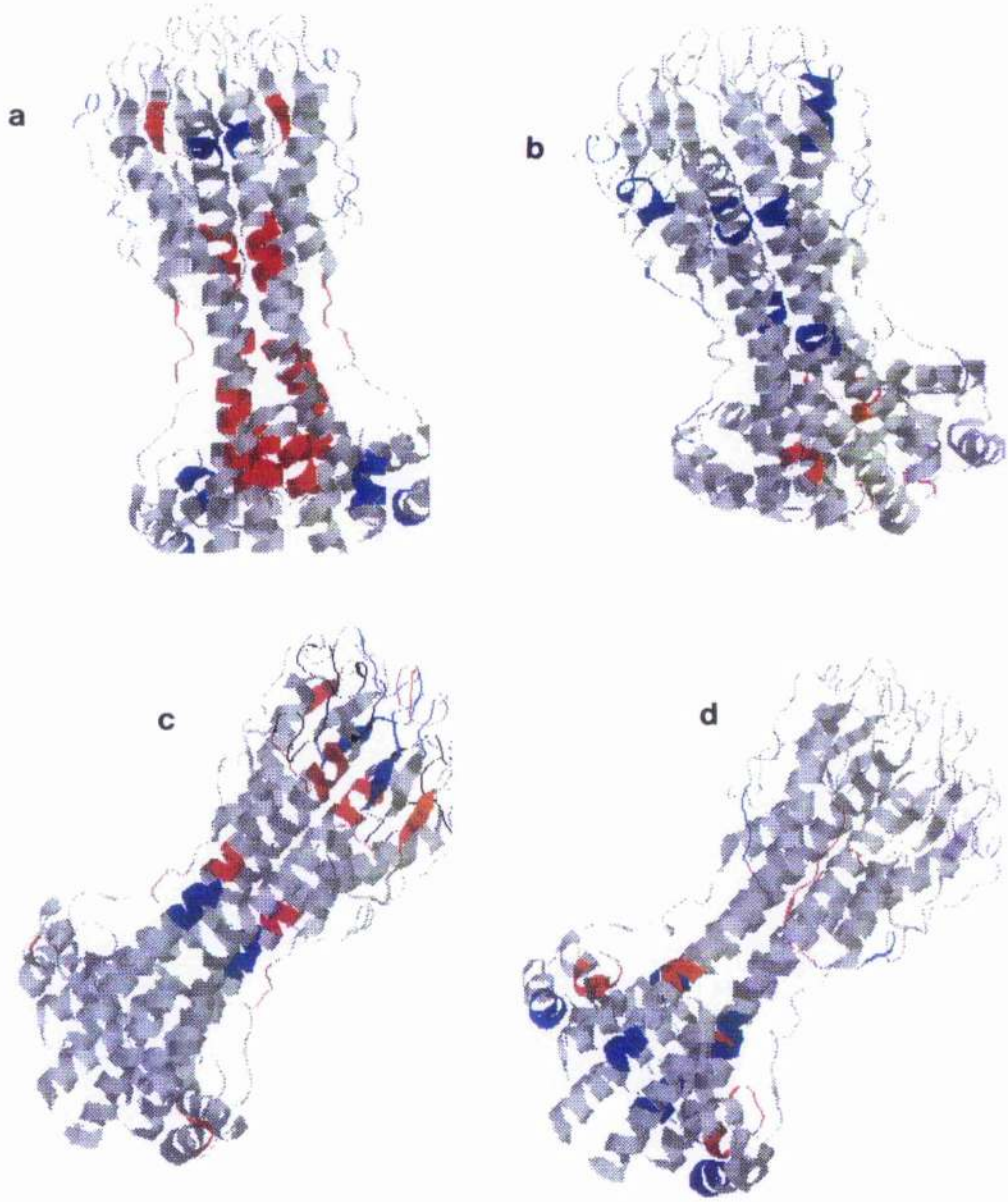


Figure 5.2. The alignment of ILTat 1.24, ILTat 1.3 and MITat 1.2 is as described by Blum *et al* (1993) and ILTat 1.22 was aligned using similar criteria. Possible CD4 epitopes are shown in bold and CD4/8 epitopes in bold and underlined.

Figure 5.2. Alignment of four VSG sequences indicating the relative positions of potential T-cell epitopes in the linear sequences.

Figure 5.3. Mapping of possible T-cell epitopes onto a tertiary VSG homodimer.



The potential T-cell epitopes highlighted in Figure 5.1. for the four amino acid sequences of MITat 1.2 (**a**), ILTat 1.22 (**b**), ILTat 1.3 (**c**) and ILTat 1.61 (**d**) were mapped onto a tertiary VSG homodimer using a computer package, "Rasmol". The potential CD4 epitopes are highlighted in red and the possible CD4/CD8 epitopes in blue.

CHAPTER 6

ANTIGENIC SPECIFICITY OF IMMUNE RESPONSES AFTER IMMUNISATION

6.1. INTRODUCTION

Results from Chapter 4 suggested that a homologous antigen-driven T-helper 1 response was generated in a trypanosome infection and that this response was also partially cross-specific to some heterologous antigen types. Similar responses could be generated by immunisation with paraformaldehyde-fixed trypanosomes. Given these results, there are two potentially important outcomes that might arise from immunisation. Firstly, that priming of a T-cell response might prevent, or at least reduce, the consequences of infection with homologous antigen type and secondly, that improved cross-specific responses to heterologous antigen types might be generated, also leading to amelioration of the course of infection.

There is remarkably little literature on immunisation against trypanosome infections but there is evidence that VAT-specific protective immune responses can be generated. Immunisation with VAT-specific monoclonal antibodies provides protection against challenge with homologous trypanosomes but not against heterologous VATs (Crowe *et al*,1984). Protection against homologous VATs can also be achieved by immunisation with irradiated trypanosomes (Wellde *et al*,1975; Pinder *et al*,1986).

My objective in this chapter was to investigate variant antigen-specific responses to immunisation and I have focused on two questions in particular: firstly, does generation

of a T-cell response to a heterologous VAT lead to a protective response against that VAT? Secondly, does immunisation with a VSG-specific peptide generate T-cell responses to the homologous VAT and to a heterologous VAT?

To address the first question, I immunised mice using paraformaldehyde-fixed trypanosomes of defined VAT as described in Chapter 4, infected them with trypanosomes of either the same or a different VAT and then monitored the fates of the infections and aspects of the T-cell response to infection. To conduct these studies I used the VATs ILTat 1.3, GUTat 7.1 and ILTat 1.61 because there was a considerable reciprocal heterologous antigen-driven response between the first two VATs but very little heterologous proliferative response between these and ILTat 1.61.

To address the second question, I made use of the theoretical considerations outlined in Chapter 5. These results indicated that it might be possible to select individual peptides, based on VSG sequences, that would generate T-cell responses against trypanosome infections. Four peptides were selected, two from the ILTat 1.3 sequence and two from the ILTat 1.61 sequence that were predicted by the "TSites" program to contain possible T-cell epitopes. All four peptides consisted of 14 amino acids with a 15th residue in each case being a cysteine residue by which they could be readily conjugated to Keyhole Limpet Haemocyanin (KLH). Amidation of the C-terminus was done for each peptide to protect the thiol group of each and prevent degradation of the peptides. Peptide 1, LAQTLDAVKATLNQC, and Peptide 4, GPTEVAKAGNEPGDC, were from the ILTat 1.3 amino acid sequence with Peptide 2, GILELGAGRAKITPC, and Peptide 3, AEAAAHLRRTTEEAC, from the ILTat 1.61 sequence. Peptides 1 and 3 were mapped to the α-helical core of the VSG homodimers whereas Peptides 2 and 4 were mapped to external loops of the VSG molecules. ILTat 1.3 and ILTat 1.61 were

the VATs selected as the basis for this study due to the lack of reciprocity in stimulating antigen-driven proliferation in the infection and immunisation regimes.

6.2. RESULTS

6.2.1. Immunisation With Paraformaldehyde-fixed Trypanosomes

6.2.1.1. Comparison Of Parasitaemias

The parasitaemia of an ILTat 1.3 infection increased steadily and peaked at day four with a parasitaemia of 1.3×10^8/ml [Figure 6.1a.]. Immunisations with ILTat 1.3 paraformaldehyde-fixed trypanosomes altered the course of an ILTat 1.3 infection, the delay in the onset of parasitaemia was five days with this group of mice being killed on day eight at 1.6×10^7/ml. In the case of a GUTat 7.1 infection after immunisation against ILTat 1.3, there was a delay in the onset of parasitaemia for two days and the rate of increase towards the peak of parasitaemia was more gradual, compared to that of the ILTat 1.3 infection, and peak occurred on day eight, five days after the ILTat 1.3 infection, at 1.3×10^8/ml [Figure 6.1a.]. At the peak of parasitaemia, >99.9% of the trypanosomes present were expressing ILTat 1.3 in the ILTat 1.3 infection, but substantially less expressed ILTat 1.3 if they were immunised with ILTat 1.3 before infection and there was also a decrease in VAT homogeneity observed with GUTat 7.1 trypanosomes infection in the ILTat 1.3-immunised mice [Table 6.1.].

The GUTat 7.1 infection reached a peak at 1.3×10^8/ml at day four of the infection [Figure 6.1b.]. Immunisation with GUTat 7.1 paraformaldehyde-fixed trypanosomes

followed by infection with GUTat 7.1 produced a delay in the detection of parasitaemia in the blood of these mice [Figure 6.1b.]. Parasites were not detected until day five. The course of the ILTat 1.3 infection after immunisation with GUTat 7.1 also resulted in a delay in detection of parasites in the bloodstream until day four and by day seven showed a peak parasitaemia of 1.3×10^8/ml. The number of trypanosomes present expressing GUTat 7.1 at the first peak of parasitaemia were >99.9% after the GUTat 7.1 infection whereas after GUTat 7.1 immunisation followed by infection with GUTat 7.1 showed a decrease in the number of trypanosomes expressing that VAT, as was the case with the trypanosomes expressing ILTat 1.3 following the ILTat 1.3 infection in GUTat 7.1-immunised mice [Table 6.1].

The parasitaemia of an ILTat 1.61 infection reached peak parasitaemia, 2.5×10^8/ml, on day four of infection. Immunisation with paraformaldehyde-fixed ILTat 1.61 trypanosomes followed by homologous infection did delay the onset of parasitaemia but only slightly and these mice were killed on day four at 1.6×10^7/ml [Figure 6.1c.]. However, the mice immunised with ILTat 1.61 then infected with ILTat 1.3 did not result in any protection against this heterologous VAT as shown by the high parasitaemia, 1.3×10^8/ml, [Figure 6.1c.] and by the homogencity of the trypanosome population at the peak of parasitaemia [Table 6.1].

6.2.1.2. Proliferative Responses Of Mononuclear Splenocytes

After immunisation with ILTat 1.3, then infection with ILTat 1.3 there was a high level of proliferation in the absence of stimulant, >10,000cpm, but after Con A stimulation the proliferative response was several fold greater at >130,000cpm [Figure 6.2a.]. There was also a high level of antigen-driven proliferation with ILTat 1.3,

GUTat 7.1 and GUTat 7.2. Heterologous antigen-driven proliferation was observed with ANTat 1.8 stimulation albeit at a lower level and a lack of heterologous proliferation with ILTat 1.61 [Figure 6.2a.]. ILTat 1.3 immunisation, with paraformaldehyde-fixed trypanosomes, followed by infection with a heterologous VAT, GUTat 7.1, resulted in similar results with an extremely high proliferative response to Con A, a substantial antigen-driven proliferative response to GUTat 7.1, ILTat 1.3 and GUTat 7.2, slightly lower level of response to ANTat 1.8 and a lack of antigen-driven proliferation when stimulated with ILTat 1.61 [Figure 6.2b.].

GUTat 7.1 immunisation followed by GUTat 7.1 challenge infection resulted in an extremely high proliferative response despite the low level of parasitaemia. The Con A response was >200,000cpm and there was a high degree of proliferation in response to GUTat 7.1 and ILTat 1.3 paraformaldehyde-fixed trypanosomes [Figure 6.2c.]. A heterologous antigen-driven proliferative response was produced with GUTat 7.2 stimulation, less with ILTat 1.61 and ANTat 1.8 stimulation. The proliferation generated after infection with ILTat 1.3 trypanosomes produced >100,000cpm with mitogenic stimulation, antigen-driven proliferation of >50,000cpm with ILTat 1.3 and >45,000cpm with GUTat 7.1 [Figure 6.2d.]. A similar degree of antigen-driven proliferation was observed with trypanosomes VATs expressing GUTat 7.2 and with ANTat 1.8 but a lack of proliferation with ILTat 1.61. These data are similar to those shown in Chapter 4 and indicate an association between the presence of a cross-reactive antigen-driven proliferative response and the cross-reactive protective response to infection.

6.2.1.3. Flow Cytometry Analyses

There was an extremely high increase in the cell numbers/spleen following immunisation with paraformaldehyde-fixed trypanosomes followed by infection [Figure 6.3]. Numbers of splenocytes in mice immunised but not infected were very similar to values from uninfected mice [Table 4.4b.] (data not shown). The number of B-cells reached a slightly higher level in the mice which were infected with VATs heterologous to the trypanosomes used in the immunisations [Figure 6.3.]. This also appeared to be the case for the T-cells and CD4 cells but not CD8 cells. These differences between homologous and heterologous infection, after immunisation, may be the result of the higher levels of parasitaemia exhibited by the heterologously challenged mice. However, the percentages of B-cells, T-cells, CD4 and CD8 cells were relatively constant when comparing these mononuclear splenocyte populations [Table 6.2.]. The ratio of CD4:CD8 cells was greater in the ILTat 1.3 immunised mice infected with ILTat 1.3 compared with those of the other three groups. The ratios of activated CD8 cells were detected as the same in each of the four cell populations examined but the ratios of activated CD4 cells differed showing that the GUTat 7.1 immunised mice infected with ILTat 1.3 having more activated CD4 cells than the equivalent mice infected with GUTat 7.1. Again, these data may be related to the higher parasitaemias detected in the mice infected with trypanosomes expressing heterologous VATs.

6.2.1.4. Cytokine Responses

Immunisation followed by infection with homologous VAT resulted in low levels of IFNγ production with antigen stimulation. Both the ILTat 1.3 immunisation followed by ILTat 1.3 infection [Figure 6.4a.] and the GUTat 7.1 immunisation followed by

GUTat 7.1 infection [Figure 6.4c.] showed that no IFNγ was detected when the mononuclear splenocytes were stimulated with medium alone but mitogen-driven IFNγ production was above 1500U/ml after 24 and 72 hours in both cases. Antigen-driven IFNγ production varied between VATs but did not exceed more than 40U/ml. When immunisation was followed by infection with the heterologous VAT, there was some IFNγ production when stimulating with medium alone and >1500U/ml with Con A stimulation [Figure 6.4b & d.]. Antigen-driven IFNγ production was detected from these mononuclear splenocytes and the levels varied between each VAT but with ILTat 1.61 production consistantly less than that of the other VATs.

In any of these four immunisation/infection regimes there was no detectable IL-5 or IL-6 production when the mononuclear splenocytes were stimulated with medium, Con A or any of the five VATs. Small amounts of IL-2 were detected after Con A stimulation but the levels were <10U/ml in each case and there was no detectable IL-2 production on antigenic stimulation.

6.2.2. Immunisation With Peptides

6.2.2.1. Comparison Of Parasitaemias

The four groups of mice immunised with the different peptide conjugates in adjuvant were infected with ILTat 1.3 and had different parasitaemias at the time of killing. This was also the case for those groups infected with ILTat 1.61. All groups were killed at the same time when the first group(s) reached peak parasitaemia, in order to compare their splenocyte responses at identical time points. ILTat 1.3-infected mice immunised with peptide 1 were killed at 1.6×10^7/ml, immunised with peptide 2 at 8×10^6/ml, with

peptide 3 at 1.3×10^8/ml and with peptide 4 at 3.2×10^7/ml. Those groups immunised prior to ILTat 1.61 infection were killed at 3.2×10^7/ml for peptide 1, at 3.2×10^8/ml for peptide 2, at 6.3×10^7/ml for peptide 3 and at 1.3×10^8/ml for peptide 4.

6.2.2.2. Proliferative Responses Of Mononuclear Splenocytes

The proliferative responses were investigated using populations from four groups of ILTat 1.61-infected mice with each having been immunised with one of four peptide conjugates before infection. The mononuclear splenocytes were stimulated with medium alone, the homologous antigen type, ILTat 1.61, and with two heterologous VATs. The homologous VAT was the positive control in these experiments. The cells from the peptide 1 immunised mice proliferated in response to homologous and one heterologous VAT, ILTat 1.3, but to a markedly lower degree with GUTat 7.1 [Figure 6.5a.].

Splenocytes from the peptide 2 immunised mice resulted in a higher level of proliferation after stimulation with medium alone, ILTat 1.61 and the heterologous VATs [Figure 6.5b.]. These cells produced a homologous antigen-driven proliferative response and a heterologous antigen-driven response with both ILTat 1.3 and GUTat 7.1.

Very high proliferative responses were detected when the cells from mice immunise with peptide 3 were stimulated with ILTat 1.61 [Figure 6.5c.]. These levels of ^{3}H-Thymidine incorporation were similar between all three groups.

The highest levels of ^{3}H-Thymidine incorporation were displayed by those cells harvested from the mice previously immunised with peptide 4 [Figure 6.5d.]. The culture medium alone produced a proliferative response >50,000cpm but there was also homologous antigen-driven and heterologous antigen-driven proliferation. Using peptide

4 for immunisations produced a superior proliferative response with ILTat 1.3 and GUTat 7.1 paraformaldehyde-fixed trypanosomes compared to the response generated with ILTat 1.61.

Taken together, these data show that immunisation with any of the four peptides generated an antigen-driven proliferative response but there was little, if any, antigenic specificity to this response, despite the peptides being chosen from unique sequence of VSGs for which the VATs generate antigen-specific responses.

6.2.2.3. Flow Cytometry Analyses

The numbers of particular cell types/spleen were determined and compared between those from mice immunised with the peptide conjugates, those immunised with the peptides and infected with ILTat 1.3 and those immunised and then infected with ILTat 1.61. In the case of peptide 1, there was an increase in the number of B-cells from mice infected with ILTat 1.3 but an even greater increase from those infected with ILTat 1.61 [Figure 6.6a.]. The number of T-cells increased following infection with either ILTat 1.3 or ILTat 1.61 compared with immunisation alone and this increase was seen by both the CD4 cells and CD8 cells. With peptide 2, there was an increase in the B-cell, T-cell, CD4 and CD8 cell populations as a result of infection with either VAT [Figure 6.6b.]. There was a several fold increase in the number of B-cells present in the spleens of mice immunised with peptide 3 and infected with ILTat 1.3 or ILTat 1.61 [Figure 6.6c.]. The numbers of T-cells also increased following trypanosome infection and this was in both the CD4 and CD8 populations with the greater increase in ILTat 1.3-infected mice than in ILTat 1.61-infected mice. With peptide 4 there was an increase in B-cell number in the ILTat 1.3-infected mice but a greater increase in the

mice infected with ILTat 1.61 trypanosomes [Figure 6.6d.]. Surprisingly perhaps, there did not appear to be any increase in the number of T-cells from the infected mice.

The proportions and ratios of cells within the mononuclear splenocyte population from four groups of mice immunised each with a different peptide conjugate without trypanosome infection were determined [Table 6.3a.]. The percentages of B-cells, T-cells, CD4 and CD8 cells did not differ too greatly between the mononuclear splenocyte populations from each group. The ratio of B:T-cells was greater in the mice immunised with peptides 1 or 2 than those immunised with peptides 3 or 4. The ratios of CD4:CD8 cells were consistent between peptides as was the proportion of activated CD4 cells within the four groups.

The main effects of infection were seen with both VATs and all four peptides. There was a decrease in the proportion of T-cells, mainly in the CD4 compartment, and this was reflected in higher B:T ratios but lower CD4:CD8 ratios.

The proportions of B-cells, T-cells, CD4 and CD8 cells, did not differ to a great extent between each group immunised with a different peptide conjugate and then infected with ILTat 1.3 [Table 6.3b.]. The various cell ratios were also very similar when comparing the four groups.

ILTat 1.61 infection, following immunisation with peptide conjugates, resulted in differing proportions and ratios of cells in the four different splenocyte populations [Table 6.3c.]. The ratio of B:T cells was similar between each of the four groups even though there was variability in the percentage of B-cells. The percentages of CD4 and CD8 cells were similar for each cell population when comparing the four mouse groups but the ratios of CD4:8 cells were variable. There appeared to be more activated CD4 cells present in the mice which were immunised with either peptides 3 or 4 than those immunised with either peptides 1 or 2.

6.2.2.4. Cytokine Responses

The mononuclear splenocyte populations harvested from the mice immunised with the peptide conjugates and then infected with either ILTat 1.3 [Figure 6.7.] or ILTat 1.61 [Figure 6.8] produced IFNγ after *in vitro* stimulation with Con A or with antigen. As had previously been seen with the proliferation data, there was no apparent antigenic specificity to the response however.

The splenocytes from the groups of mice immunised with the peptide conjugates and then infected with ILTat 1.3 trypanosomes produced differing amounts of IL-2 when stimulated with Con A [Figure 6.9a.]. The splenocytes from mice immunised with peptide 3 produced slightly higher levels of IL-2 than the other three. This could be attributed however to the higher parasitamia detected in this group of mice. There was no detectable IL-2 when these cells from the four immunised groups were stimulated *in vitro* either with culture medium or with the three VATs used. There was no detectable IL-5 production by these mononuclear splenocytes when stimulating with medium alone, Con A or ILTat 1.3, ILTat 1.61 or GUTat 7.1 paraformaldehyde-fixed trypanosomes.

In general, the IL-2 production from the immunised mice infected with ILTat 1.61 trypanosomes was higher than those from the ILTat 1.3-infected mice [Figure 6.9b.]. As also found in the ILTat 1.3-infected mice, there was no detectable IL-2 production when these mononuclear splenocytes were *in vitro* stimulated with medium or any of the three VATs. The splenocytes from these four groups of mice failed to produced any detectable IL-5 following either medium, mitogen or trypanosome antigen stimulation.

6.3. DISCUSSION

It was possible to partially immunise mice using paraformaldehyde-fixed trypanosomes expressing a single VAT. These mice were substantially protected against homologous challenge and there was also a degree of cross-protection towards heterologous challenge. This was reflected by monitoring parasitaemias and in immunofluorescence studies which showed that a high percentage of the trypanosomes detected at the first peak of parasitaemia did not express the VSG surface coat of those trypanosomes used for the original inoculum. In contrast, the mice immunised with peptides and adjuvant provided only a slight degree of protection as indicated by the different parasitaemias of each group. Hindsight suggests that twice as many mice should have been immunisated with each peptide with half of these mice being allowed to reach the first parasitaemic peak, however long it took, and then examining the proliferative and cytokine responses, rather than killing all mice when one group reached parasitaemic peak. This would have allowed comparison of responses when all groups involved were at the first peak of parasitaemia as well as data regarding the degree of protection against challenge with homologous and heterologous VATs. The experiments conducted, suggest that there was greater protection against homologous and heterologous trypanosomes when prior immunisation was with paraformaldehyde-fixed trypanosomes than with peptide conjugates with adjuvant. These data may reflect that these selected peptides are not very immunogenic and that others should have been selected, that more than two immunisations should have been given or that mice of a different genetic background might usefully have been explored. The key to successful immunisation may be to have the trypanosomes as intact as possible before the

immunisation and the degree of protection may be increased further if the trypanosomes were immunised with adjuvant.

Perhaps the most significant finding from this section of my work was that a measure of cross-protection was engendered by immunisation with one VAT against a second VAT. This cross-protection was associated with a heterologous antigen-driven proliferative T_H1 response but there was no cross-protection against another VAT for which there was very little evidence of a heterologous antigen-driven T-cell response. The implication from this finding is that immunisation to generate T-cell responses that are not VAT-specific is possible and could potentially overcome, at least in part, the VAT-specificity of antibody responses. Unfortunately, there has been little other work conducted on immunisation against trypanosomiasis with which this result can be compared.

Irradiated trypanosomes have been used in a number of studies to provide protection against trypanosome infections. Rats immunised with irradiated trypanosomes provided protection following challenge with homologous trypanosomes but there was no protection against heterologous challenge (Wellde *et al*, 1975). This was also observed in mice, but doses lower than 10^6 trypanosomes/mouse resulted in less protection against homologous infection (Campbell & Phillips, 1976). Athymic mice and their heterozygous littermates, immunised with irradiated trypanosomes, survived for >25 days longer than non-immunised mice following homologous challenge (Campbell *et al*, 1978). Although irradiation has proved successful in the past as a method of immunisation, it is far easier to use paraformaldehyde-fixed trypanosomes than 60,000 rads to achieve essentially the same result. A significant degree of protection was shown when mice were intravenously immunised with formaldehyde-treated infected blood yet subcutaneous immunisation did not provide protection (Herbert & Lumsden, 1968). Herbert &

Lumsden (1968) could not achieve cross-protection when they immunised with one VAT but when a cocktail of four different VATs, using formaldehyde-fixed infected blood, was used for immunisation then the mice were protected against challenge with each of the four trypanosome VATs. This study involved immunisation with four VATs to protect against these four VATs whereas I could achieve a degree of protection against at least two VATs with an immunisation of a single VAT. Purified VSG, dissolved in saline, also provided protection against homologous but not heterologous infections when as little as 3μg of VSG was used for immunisation purposes but even 10 times this amount did not protect against heterologous challenge (Baltz *et al*,1977). Protection against *T.brucei* has also been transferred with immune sera, produced either by drug cure or immunisation, and also by adoptive transfer of splenocytes from immunised mice but the VAT specificity of response was not investigated (Takayanagi & Enriquez,1973; Campbell & Phillips,1976).

The majority of investigations involving immunisations against trypanosome infections have involved monitoring parasitaemias and/or taking serum samples at timepoints throughout the immunisation and infection regimes. Very little work has examined the proliferative T-cell responses or the cytokine production. I have shown a strong proliferative and IFNγ response was achieved following immunisations with paraformaldehyde-fixed trypanosomes and then challenge with homologous trypanosomes and a degree of cross-protection against heterologous challenge suggesting that there are common epitopes between those VATs. In the view of the absence of specific antibodies detected against the heterologous VAT's examined following immunisation with paraformaldehyde-fixed trypanosomes (Chapter 4), this strongly suggests a role for T-cells in the protection against African trypanosome infections.

Isolating common epitopes between VSG sequences using the "TSites" program, as previously described (Chapter 5), proved difficult even though the algorithms used in this program have been successfully used before. Incomplete Freund's adjuvant and peptides 20 amino acids in length from *M.tuberculosis* for example successfully induced T-cell proliferation *in vitro* (Ashbridge *et al*,1992). The immunogenicity of the peptides would have had an influence on the immune response as previously shown (Panina-Bordignan *et al*,1989; Vordermeier *et al*,1993) as well as the detailed amino acid composition of the peptides as shown by HIV (Boehncke *et al*,1993) and Hepatitis B (Bertoletti *et al*,1994) research. Interaction with the peptide, T-cell receptor and MHC complex may occur but the lack of costimulation will prevent an effective immune response being generated. The peptide selected therefore should be one which will bind with MHC molecules and have T-cells which will recognise it and respond effectively. Although proliferative and cytokine responses were generated following peptide immunisations and then infection, it may have been possible to delay the onset of parasitaemia and generate a more effective proliferative and IFNγ response if further immunisations had been given or if different peptides were selected. It would also have been beneficial if a mitogen-driven prolifcrative response was included as an extra positive control in addition to the homologous antigen control.

Based on the idea of identification of a peptide that generated responses protective against multiple VATs, a further development can be envisaged, beyond the scope of this project to investigate: DNA vaccination. This type of vaccination would introduce a partial VSG gene sequence resulting in peptide production with the peptides being recognised as foreign by the host immune system. If the DNA is supercoiled then it can be injected into the skin or muscle of the host with the skin cells being the better choice as there is a reduced risk of disrupting the hosts normal gene functions due to the quick

turnover of skin cells (Simmonds *et al*,1997). DNA vaccines have proven successful in malaria infections using the sequence for the circumsporizoite protein; in tuberculosis using the gene for a heat-shock protein; and in the SV40 tumour model using the SV40 large tumour antigen (reviewed by Simmonds *et al*,1997).

In conclusion, I have shown that it is possible to achieve protection against *T.brucei* infections by one immunisation procedure and responses indicative of protection with a second. Not only was it possible to protect against homologous challenge but more importantly it was possible to achieve partial cross-protection against a heterologous VAT. Although this was a useful step forward more work will be required to determine how many VATs can be protected against by an immunisation containing a single VAT.

Figure 6.1a. Parasitaemias of ILTat 1.3 and GUTat 7.1 infections after immunisation of mice with paraformaldehyde-fixed ILTat 1.3 trypanosomes.

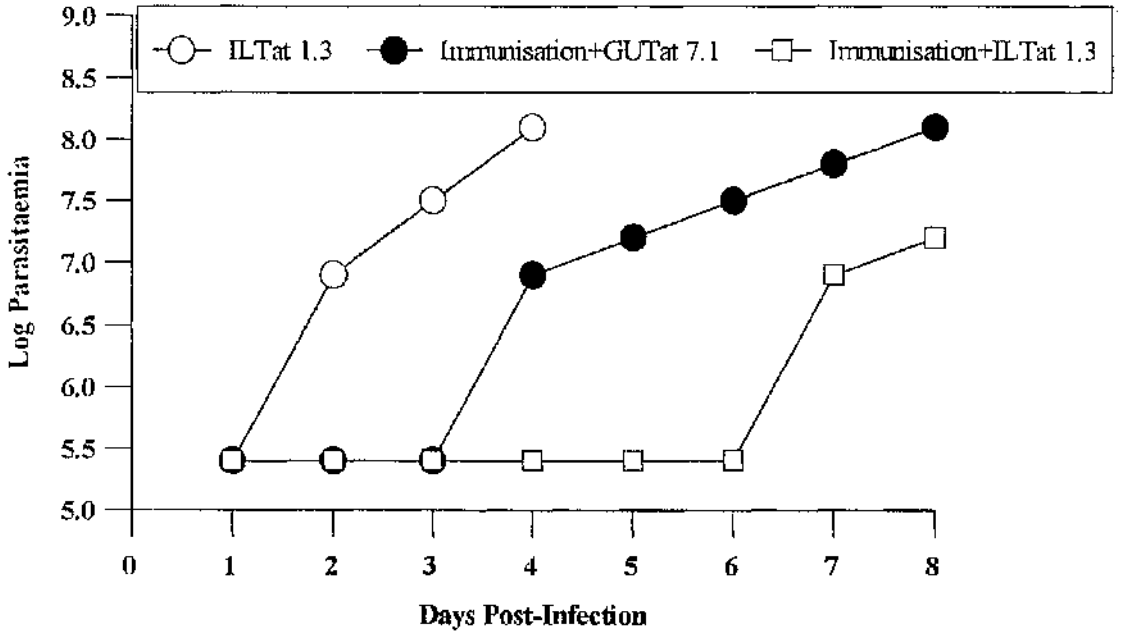


Figure 6.1b. Parasitaemias of GUTat 7.1 and ILTat 1.3 infections after immunisation of mice with paraformaldehyde-fixed GUTat 7.1 trypanosomes.

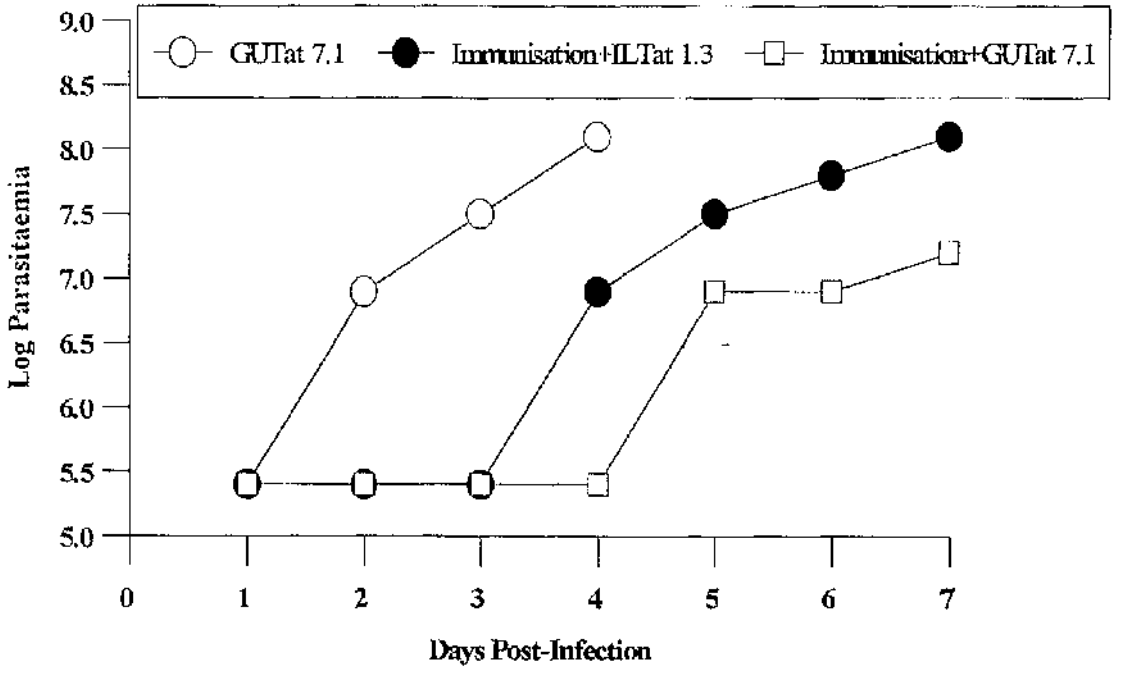


Figure 6.1c. Parasitaemias of ILTat 1.61 and ILTat 1.3 infections after immunisation of mice with paraformaldehyde-fixed ILTat 1.61 trypanosomes.

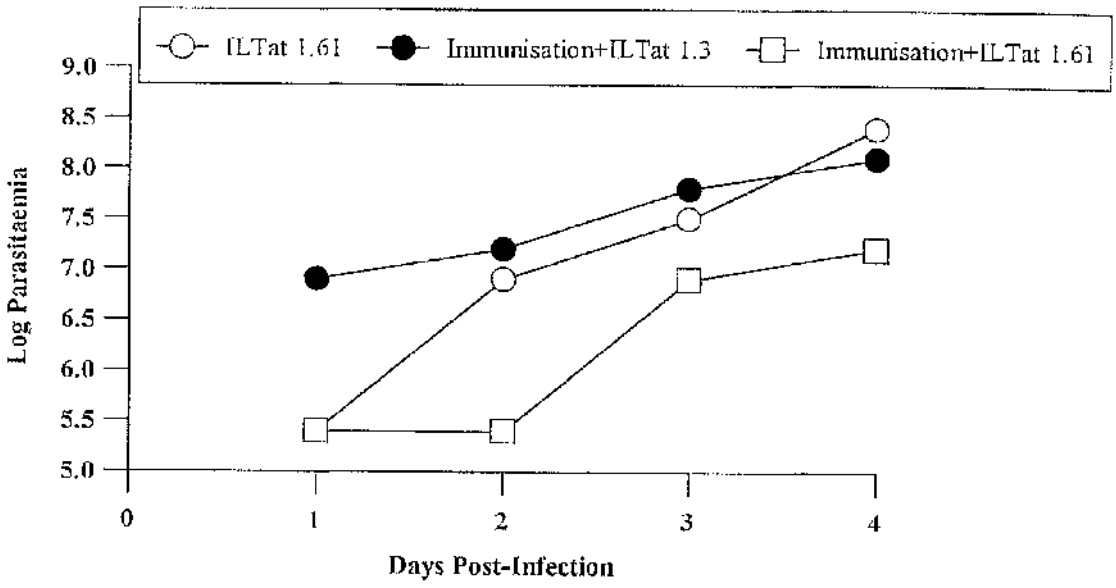


Figure 6.1. Parasitaemias were determined daily by the 'rapid matching' method from groups of infected mice (controls) and from groups which were immunised with 10^6 paraformaldehyde-fixed trypanosomes expressing either ILTat 1.3 (**a**), GUTat 7.1 (**b**) or ILTat 1.61 (**c**). Two weeks after immunisation, groups of mice were infected with homologous antigen type or infected with the heterologous VAT using 10^4 trypanosomes/mouse.

Table 6.1. Percentage homogeneity of trypanosome population at the first peak of parasitaemia in mice immunised then infected.

Immunising VAT	VAT used for infection	Percentage homogeneity of population
None	ILTat 1.3	>99.9
ILTat 1.3	ILTat 1.3	20
ILTat 1.3	GUTat 7.1	70
None	GUTat 7.1	>99.9
GUTat 7.1	GUTat 7.1	35
GUTat 7.1	ILTat 1.3	75
None	ILTat 1.61	>99.9
ILTat 1.61	ILTat 1.61	54
ILTat 1.61	ILTat 1.3	>99.9

Percentage homogeneity of the trypanosome population for VAT expression was determined at the time of killing for each group using the immunofluorescence antibody technique on blood smears. These groups of mice were first immunised with 10^6 paraformaldehyde-fixed trypanosomes expressing ILTat 1.3, GUTat 7.1 or ILTat 1.61 and then infected two weeks later with 10^4 trypanosomes/mouse of either homologous or heterologous VAT.

Figure 6.2. Proliferative responses of mononuclear splenocytes were examined after immunisation with 10^6 paraformaldehyde-fixed trypanosomes/mouse expressing ILTat 1.3 then infected with 10^4 trypanosomes/mouse using lines expressing ILTat 1.3 (**a**) or GUTat 7.1 (**b**) trypanosomes. The reciprocal immunisation/infection regime was also examined with mice being immunised against GUTat 7.1 and then infected with GUTat 7.1 (**c**) or ILTat 1.3 (**d**). The cells were stimulated *in vitro* with medium alone, Con A (8μg/ml) or one of five different VATs. The results are expressed as the geometric means ± 2 SE.

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Figure 6.2a. Proliferative responses of mononuclear splenocytes after ILTat 1.3 immunisation followed by ILTat 1.3 infection.

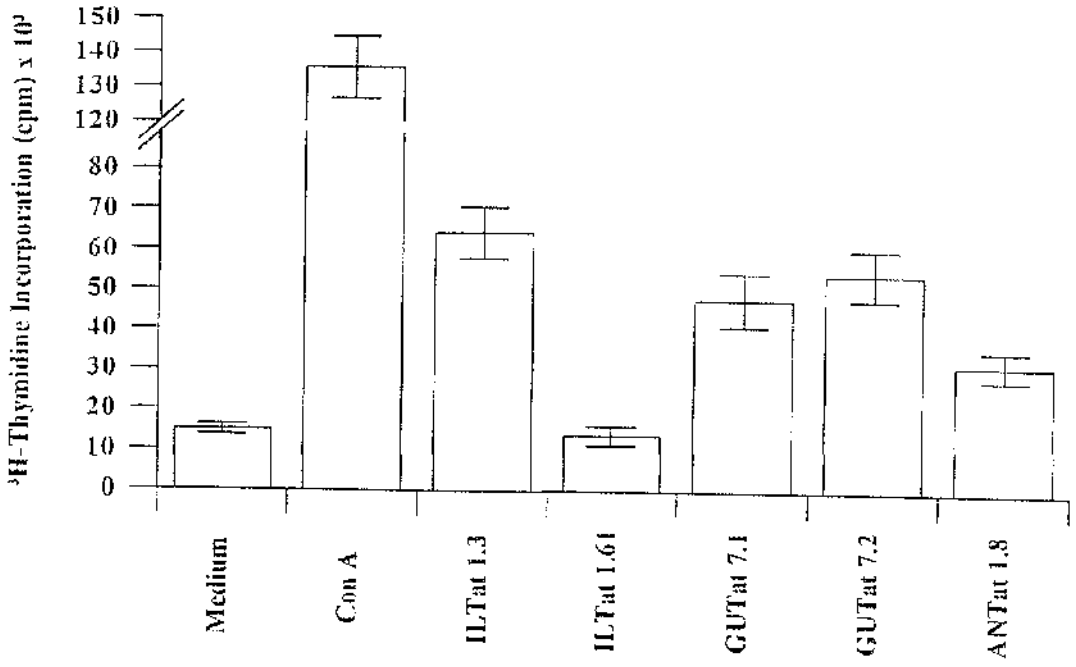


Figure 6.2b. Mononuclear splenocyte proliferative responses from mice immunised with ILTat 1.3 then infected with GUTat 7.1 trypanosomes.

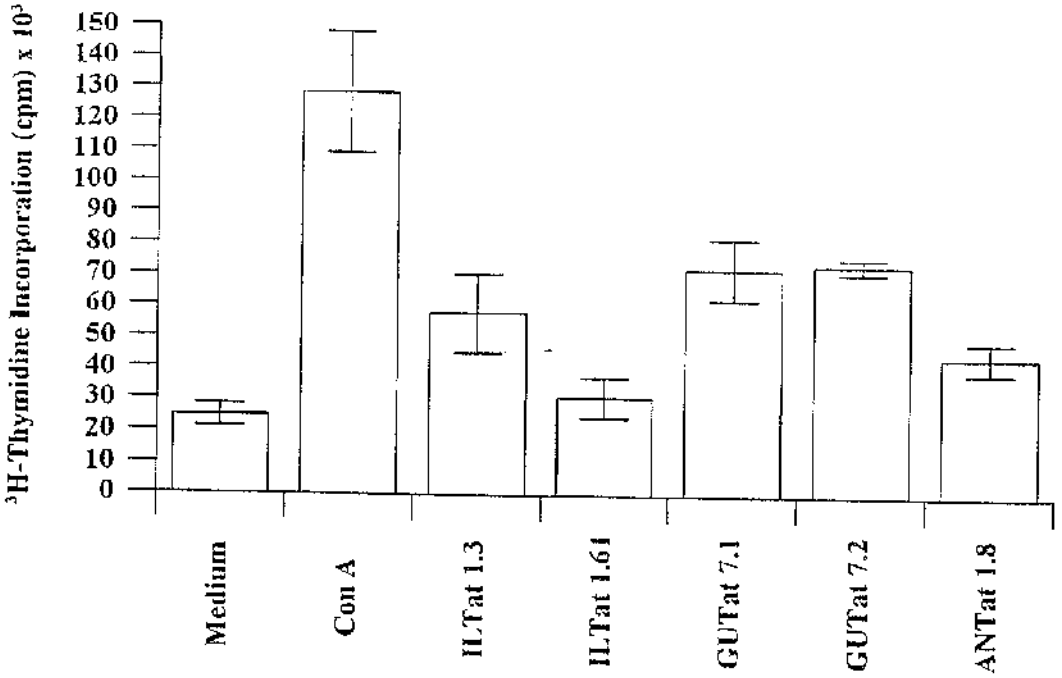


Figure 6.2c. Proliferative responses of the mononuclear splenocyte population after GUTat 7.1 immunisation and GUTat 7.1 infection.

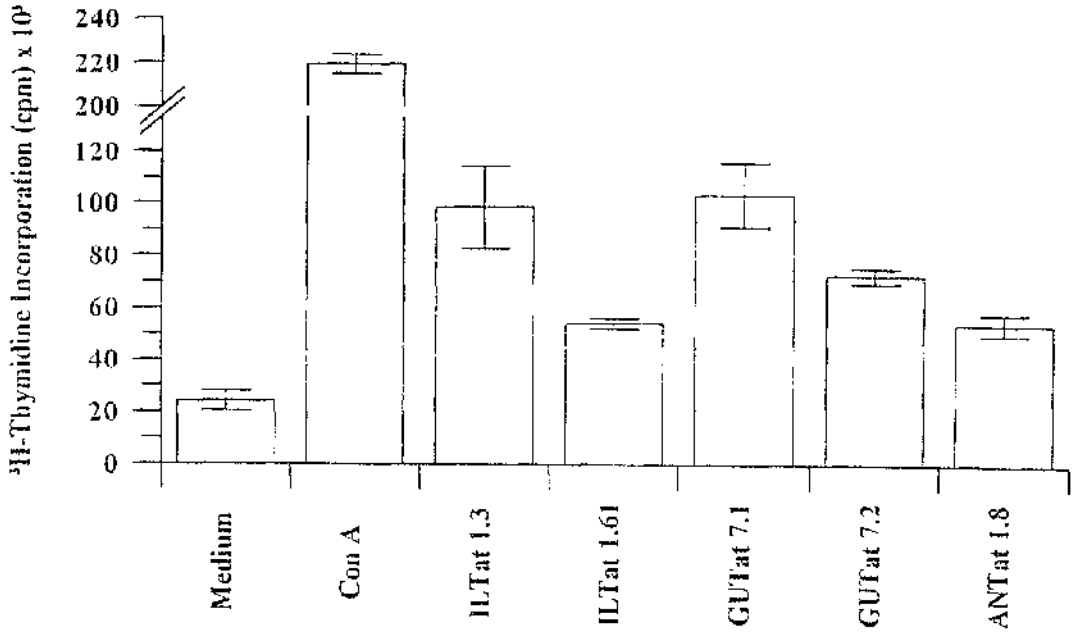


Figure 6.2d. Mononuclear splenocyte proliferation after GUTat 7.1 immunisation then infection with ILTat 1.3.

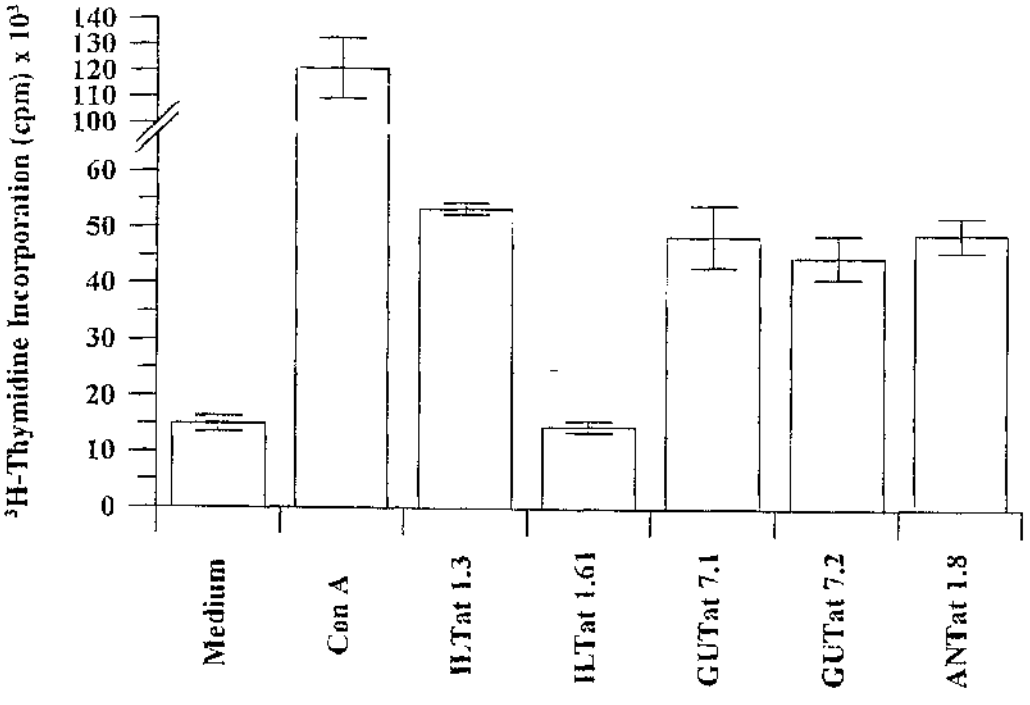
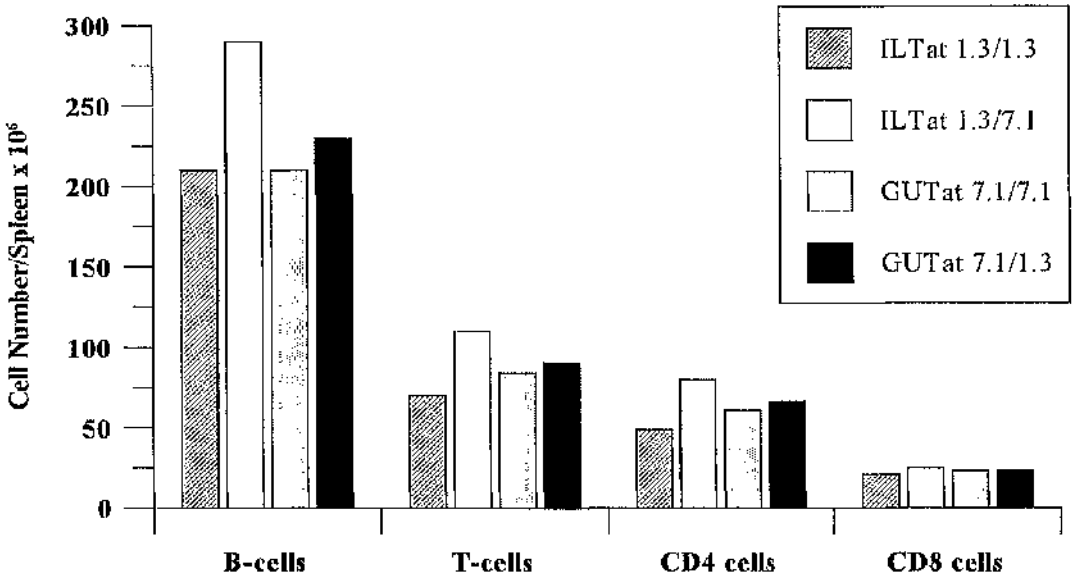


Figure 6.3. Flow cytometry analyses of mononuclear splenocytes following immunisation of mice with fixed trypanosomes then infection with trypanosomes.



Mononuclear splenocytes were purified and labelled for CD19 (B-cells), CD3 (T-cells), or double labelled for CD4 and CD8 and the cell number/spleen for each population determined by FACS analysis. The mean of 5 mice/group are shown with 5000 cells counted for each sample. In the key box, the VAT used for immunisation is indicated first, followed by the VAT used for infection, for each group of mice.

Table 6.2. Percentages and ratios of cell populations of mononuclear splenocytes following immunisation and infection regimes.

Cell Type	ILTat 1.3 / ILTat 1.3	ILTat 1.3 / GUTat 7.1	GUTat 7.1 / GUTat 7.1	GUTat 7.1 / ILTat 1.3
B	59	57	55	60
T	20	21	22	23
CD4	14	16	16	17
CD8	6	5	6	6
B:T ratio	3:1	2.6:1	2.5:1	2.6:1
CD4:8 ratio	2.3:1	3.2:1	2.7:1	2.8:1
CD4:25 ratio	25:1	25:1	33:1	20:1
CD8:25 ratio	100:1	100:1	100:1	100:1

Percentages and ratios were determined from FACS analyses of the cell populations from groups of five immunised then infected mice. CD25 was used as a marker of activation. In the header for each column, the VAT used for immunisation is shown first, followed by the VAT used for infection.

Figure 6.4. IFNγ production was measured from the mononuclear splenocyte populations after immunisation with ILTat 1.3 followed by infection with either ILTat 1.3 (**a**) GUTat 7.1 (**b**) and also after immunisation with GUTat 7.1 followed by infection with either GUTat 7.1 (**c**) or ILTat 1.3 (**d**). The cells were either stimulated with medium alone, Con A (8μg/ml) or one of five different antigen types and the samples were analysed either 24 hours (white) or 72 hours (black) later. The results are expressed as the means of triplicate wells minus the background readings, that is the readings obtained from the wells without any sample.

Figure 6.4a. IFNγ production from mononuclear splenocytes after ILTat 1.3 immunisation and infection.

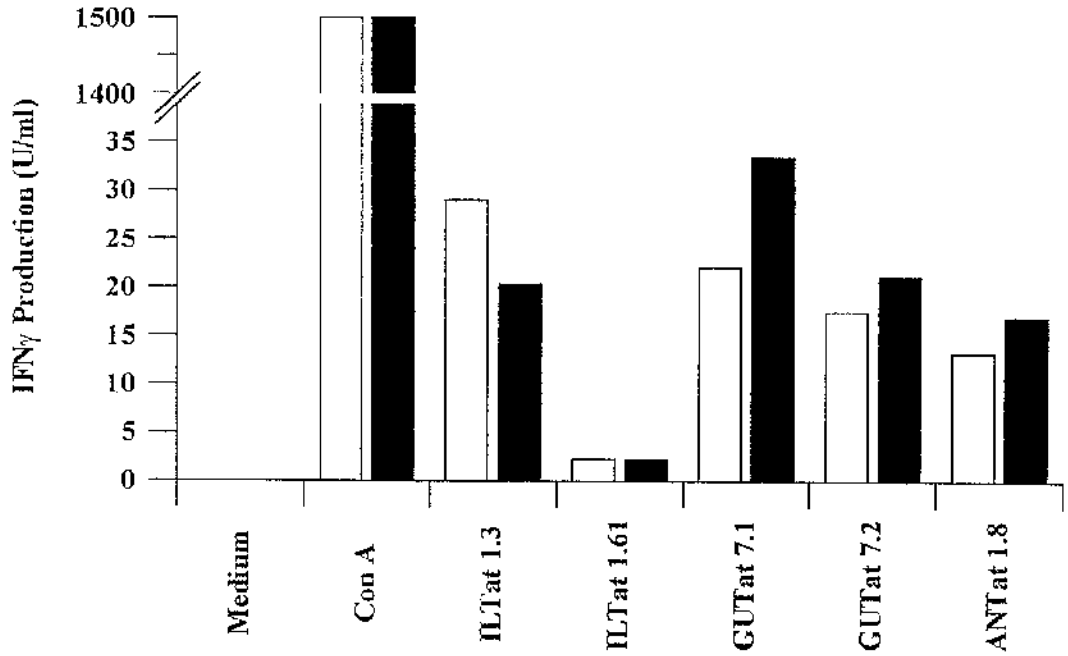


Figure 6.4b. IFNγ production from mononuclear splenocytes after ILTat 1.3 immunisation then GUTat 7.1 infection.

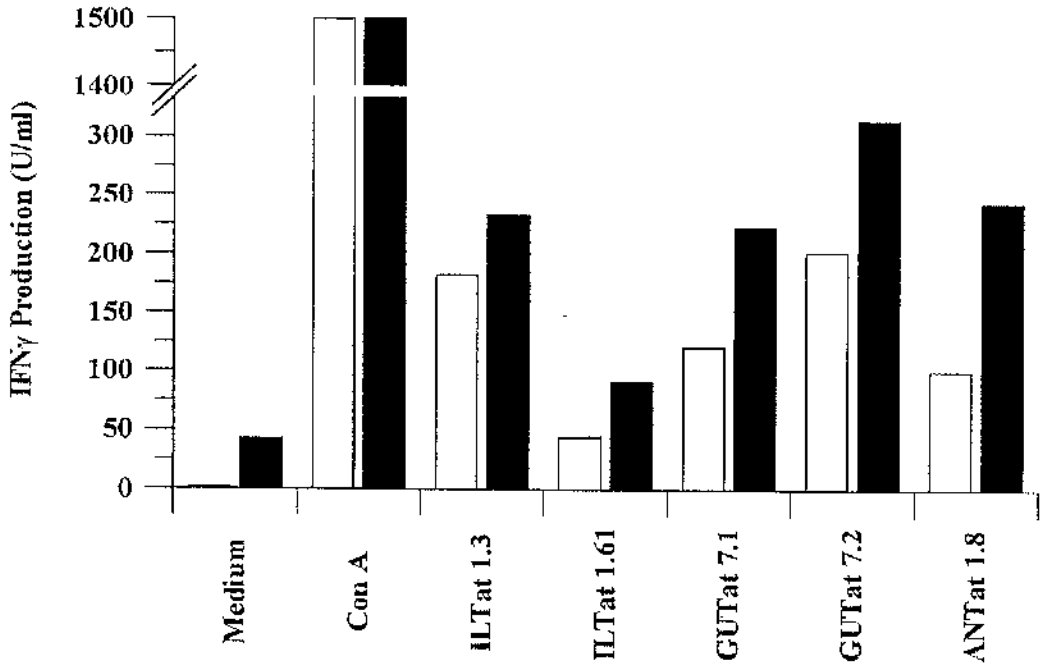


Figure 6.4c. IFNγ production from mononuclear splenocytes after GUTat 7.1 immunisation then infection.

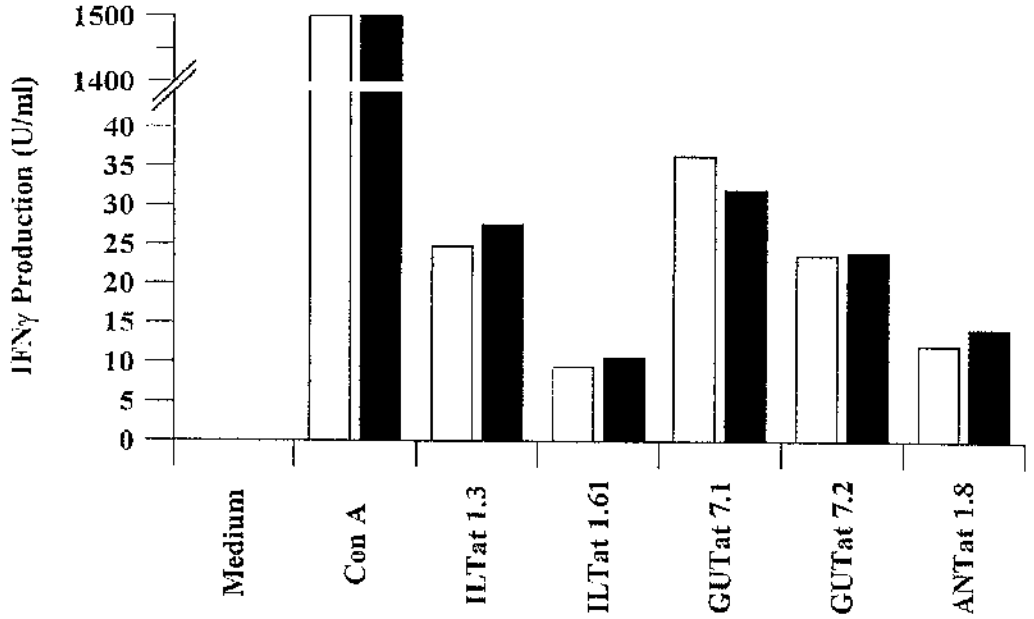


Figure 6.4d. Production of IFNγ by mononuclear splenocytes following GUTat 7.1 immunisation then infection with ILTat 1.3 trypanosomes.

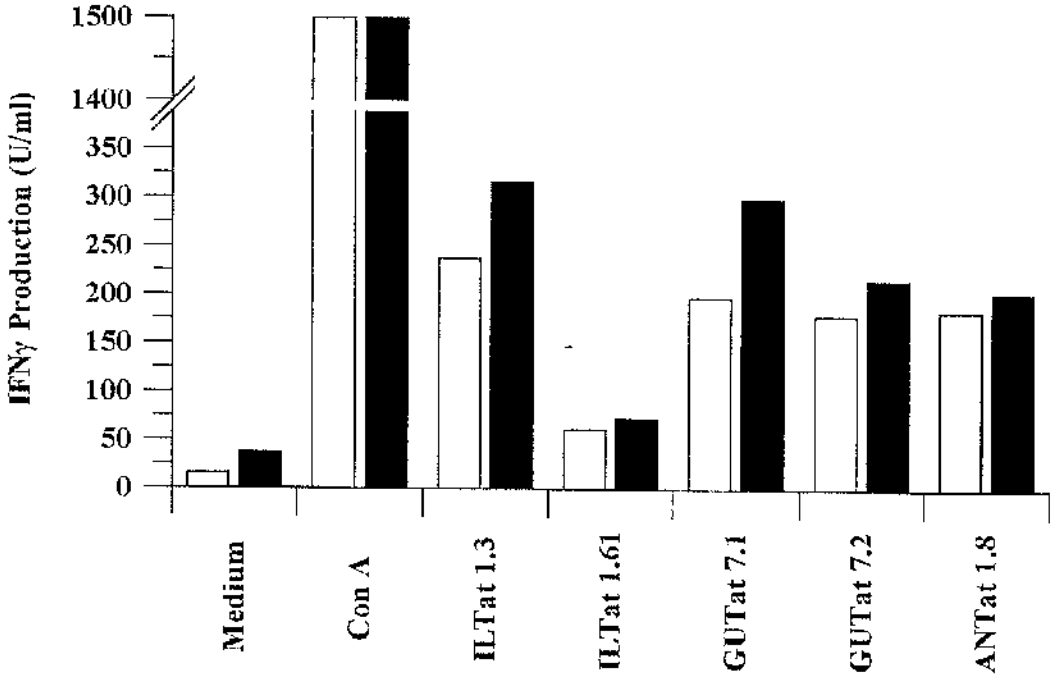


Figure 6.5. The proliferative responses of the mononuclear splenocyte populations was examined using mice immunised with peptides conjugated to adjuvant and then infected with ILTat 1.61 trypanosomes. Each of the four groups of mice were either immunised with peptide 1 (**a**), peptide 2 (**b**), peptide 3 (**c**) or peptide 4 (**d**) and all mice were killed when peak parasitaemia (1.3×10^8 trypanosomes/ml) was reached by at least one of the groups. Stimulation *in vitro* was with medium alone or with one of three different paraformaldehyde-fixed trypanosome VATs. The results are expressed as the mean values of quadruplicate wells ± 2 SE. The sequence for peptide 1 was derived from the α helix ('A' region) of ILTat 1.3 VSG; the sequence for peptide 2 from the 'g' loop of ILTat 1.61 VSG; the sequence for peptide 3 from the α helix ('H' region) of ILTat 1.61 VSG and the sequence for peptide 4 from the 'i' loop of the ILTat 1.3 VSG.

Figure 6.5a. Proliferative responses of mononuclear splenocytes from mice immunised with peptide 1 and infected with ILTat 1.61.

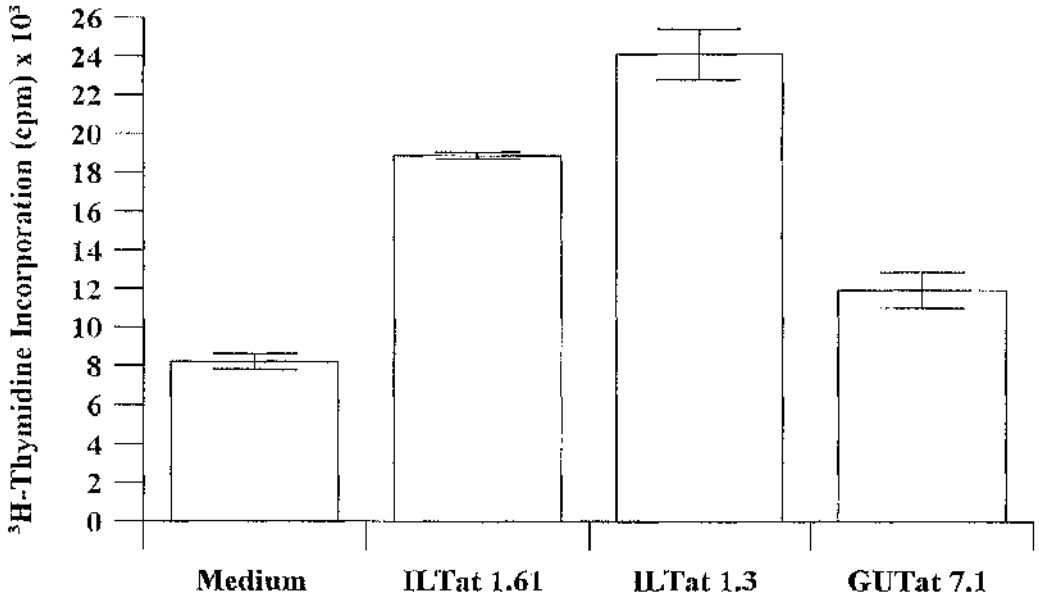


Figure 6.5b. Proliferation of mononuclear splenocyte populations following immunisation with peptide 2 and ILTat 1.61 infection.

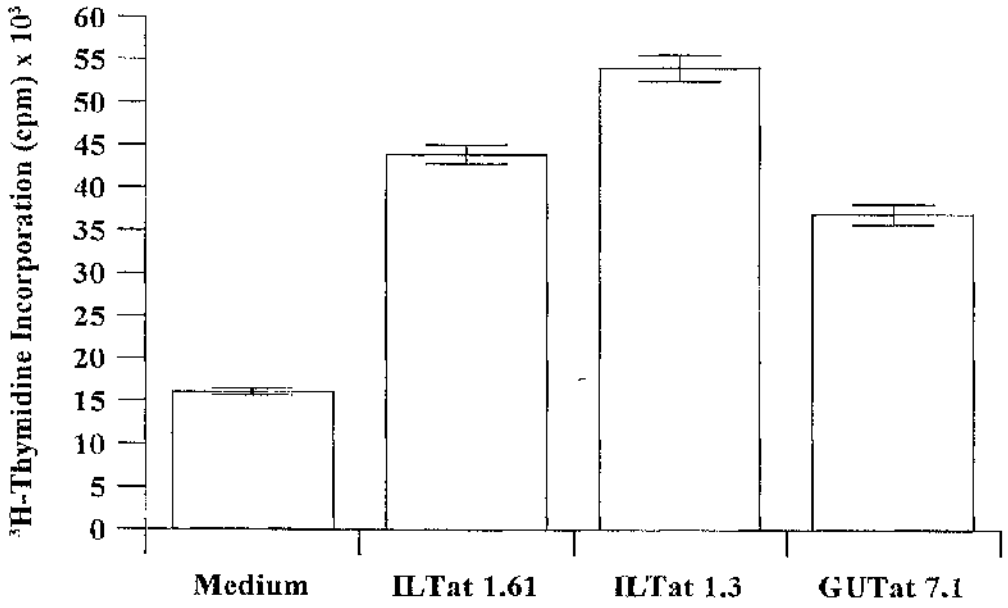


Figure 6.5c. Proliferation of mononuclear splenocytes following immunisation with peptide 3 and ILTat 1.61 infection.

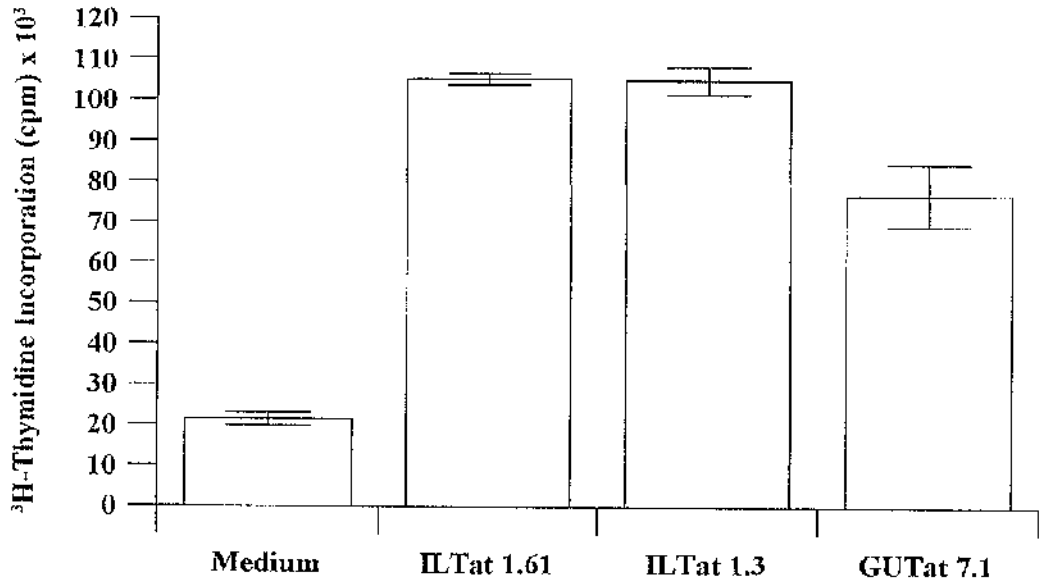


Figure 6.5d. Proliferative responses of mononuclear splenocyte populations after immunisation with peptide 4 and infection with ILTat 1.61.

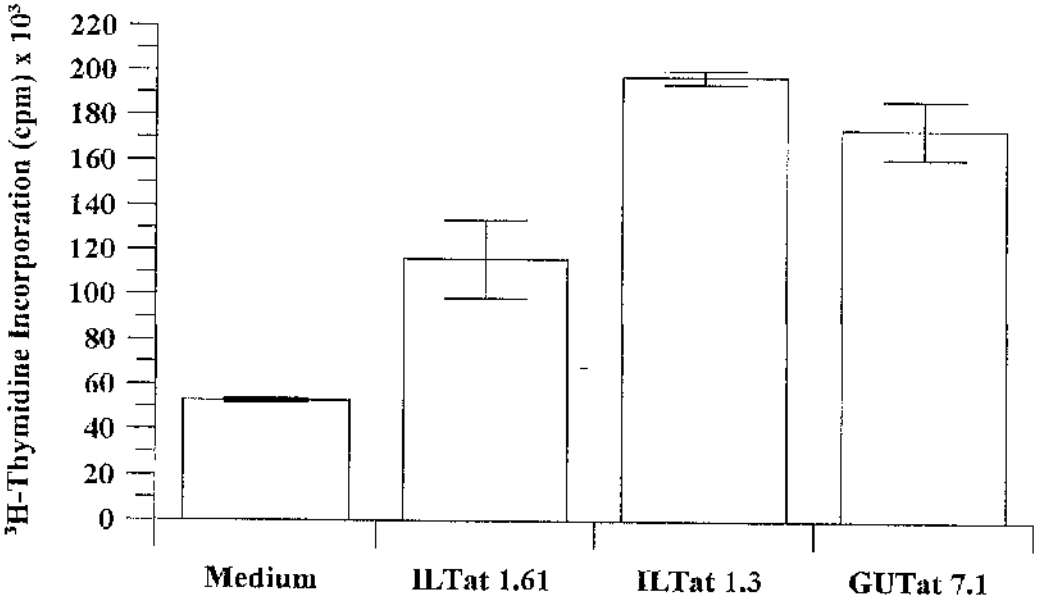


Figure 6.6. The cell numbers/spleen were determined for a number of populations by flow cytometry and compared between those from mice immunised with peptides, those which were immunised and then infected with ILTat 1.3 and those immunised and infected with ILTat 1.61 trypanosomes. The groups were immunised with either peptide 1 (**a**), peptide 2 (**b**), peptide 3 (**c**) or with peptide 4 (**d**) conjugated to a KLH carrier and injected with adjuvant. The populations were counted for B-cell, T-cells, CD4 cells and CD8 cells with 5000 cells counted in each sample analysed.

Figure 6.6a. FACS analyses of mononuclear splenocytes after immunisation with peptide 1 and infection with different trypanosome lines.

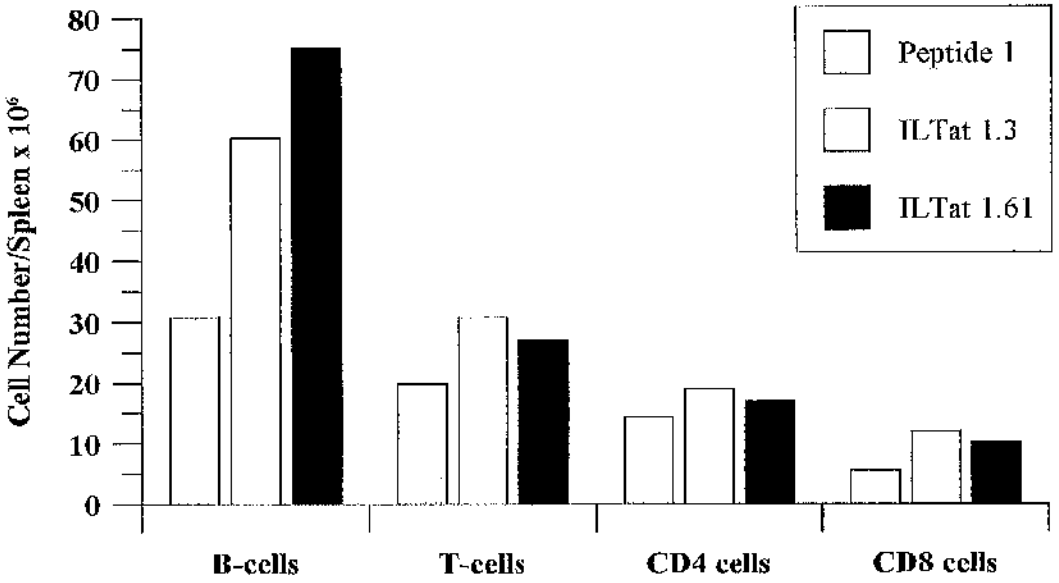


Figure 6.6b. FACS analyses of mononuclear splenocytes after immunisation with peptide 2 and infection with different trypanosome lines.

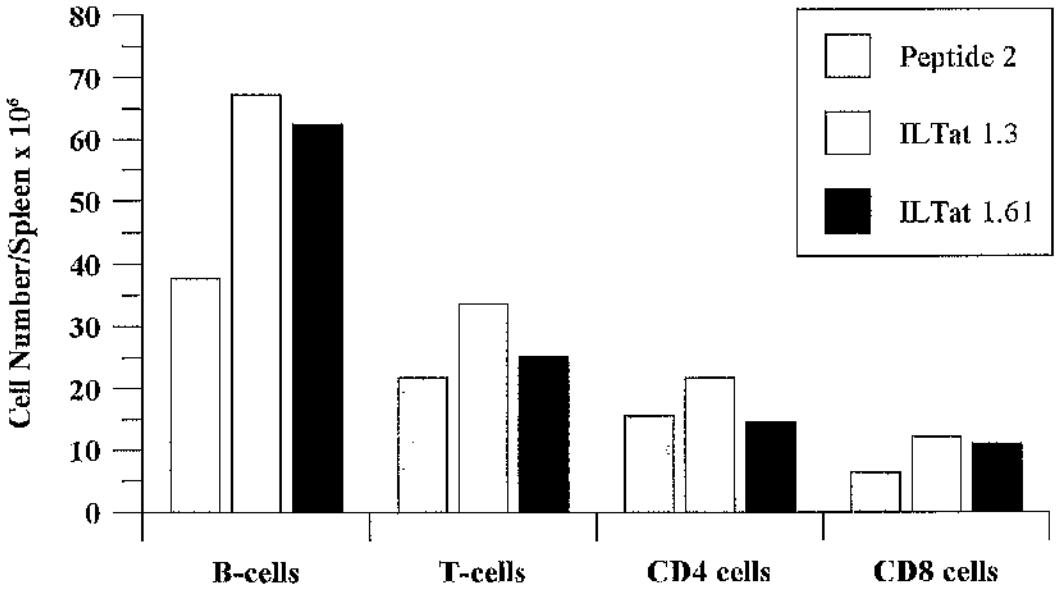


Figure 6.6c. Flow cytometry examination of mononuclear splenocyte populations following immunisation with peptide 3 and infection with trypanosomes.

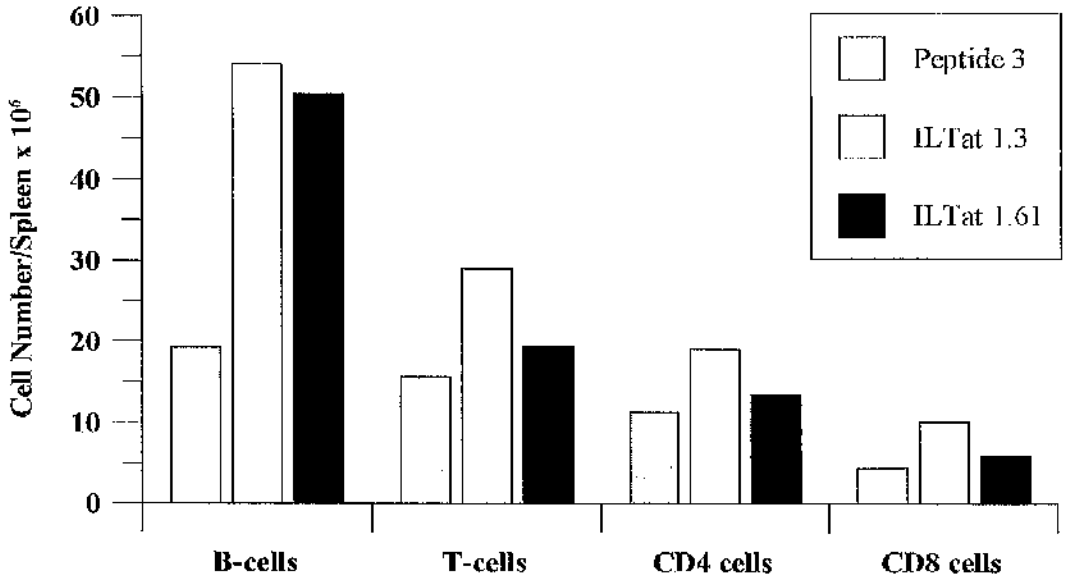


Figure 6.6d. FACS analyses of mononuclear splenocytes after immunisation with peptide 4 and infection with different trypanosome lines.

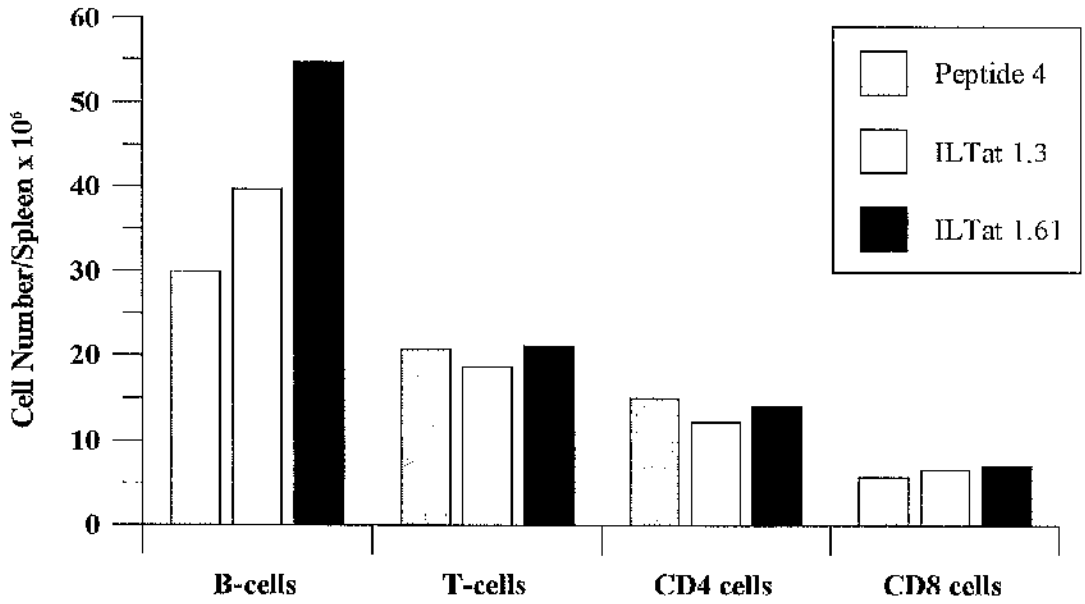


Table 6.3. The percentages and ratios of cell populations were determined by FACS analyses of mononuclear splenocyte populations using groups of mice in different immunisation and infection regimes. These analyses were on populations immunised with the four peptides (**a**), on populations immunised with the peptides then infected with ILTat 1.3 trypanosomes (**b**) and on cells from immunised mice which were then infected with ILTat 1.61 (**c**). The values are expressed as percentages except where otherwise stated. CD25 was used as a marker of activation and 5000 cells were counted from each sample.

ND = no stained cells detected.

Table 6.3a. Percentages and ratios of mononuclear splenocytes following immunisation with peptide conjugates.

Cell Type	Peptide 1	Peptide 2	Peptide 3	Peptide 4
B	56	54	48	52
T	36	31	39	36
CD4	26	22	28	26
CD8	10	9	11	10
B:T ratio	1.6:1	1.7:1	1.2:1	1.4:1
CD4:8 ratio	2.6:1	2.4:1	2.5:1	2.6:1
CD4:25 ratio	50:1	50:1	50:1	50:1
CD8:25 ratio	ND	ND	ND	ND

Table 6.3b. Flow cytometry examination to determine proportions of mononuclear splenocyte populations after immunisation with peptides and ILTat 1.3 infection.

Cell Type	Peptide 1	Peptide 2	Peptide 3	Peptide 4
B	51	56	54	55
T	26	28	29	26
CD4	16	18	19	17
CD8	10	10	10	9
B:T ratio	2:1	2:1	1.9:1	2.1:1
CD4:8 ratio	1.6:1	1.8:1	1.9:1	1.9:1
CD4:25 ratio	25:1	25:1	20:1	25:1
CD8:25 ratio	100:1	100:1	100:1	100:1

Table 6.3c. FACS analyses of mononuclear splenocytes to determine cell proportions following immunisation with peptides then ILTat 1.61 infection.

Cell Type	Peptide 1	Peptide 2	Peptide 3	Peptide 4
B	67	52	60	62
T	24	21	23	24
CD4	15	12	16	16
CD8	9	9	7	8
B:T ratio	2.8:1	2.5:1	2.6:1	2.6:1
CD4:8 ratio	1.7:1	1.3:1	2.3:1	2:1
CD4:25 ratio	33:1	33:1	25:1	25:1
CD8:25 ratio	100:1	100:1	100:1	100:1

Figure 6.7. The production of IFNγ from mononuclear splenocyte populations after immunisation, with one of four peptides then infection with ILTat 1.3 trypanosomes, was measured. The cells were purified from mice that had been immunised with either peptide 1 (**a**), peptide 2 (**b**), peptide 3 (**c**) or peptide 4 (**d**). The supernatants were harvested 24 (white) and 72 (black) hours after *in vitro* stimulation with medium, Con A (8μg/ml) and three different trypanosome VATs. The results are expressed as the means of triplicate wells minus the background, that is the readings obtained from the wells without any sample.

Figure 6.7a. Production of IFNγ from mononuclear splenocytes after immunisations with peptide 1 and infection with ILTat 1.3.

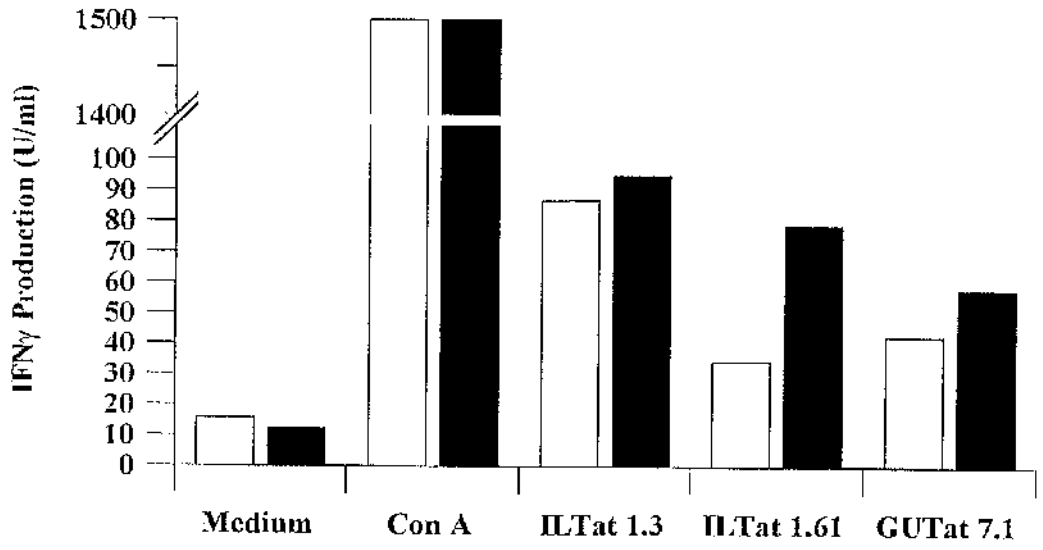


Figure 6.7b. IFNγ production following immunisation with peptide 2 and infection with ILTat 1.3 trypanosomes.

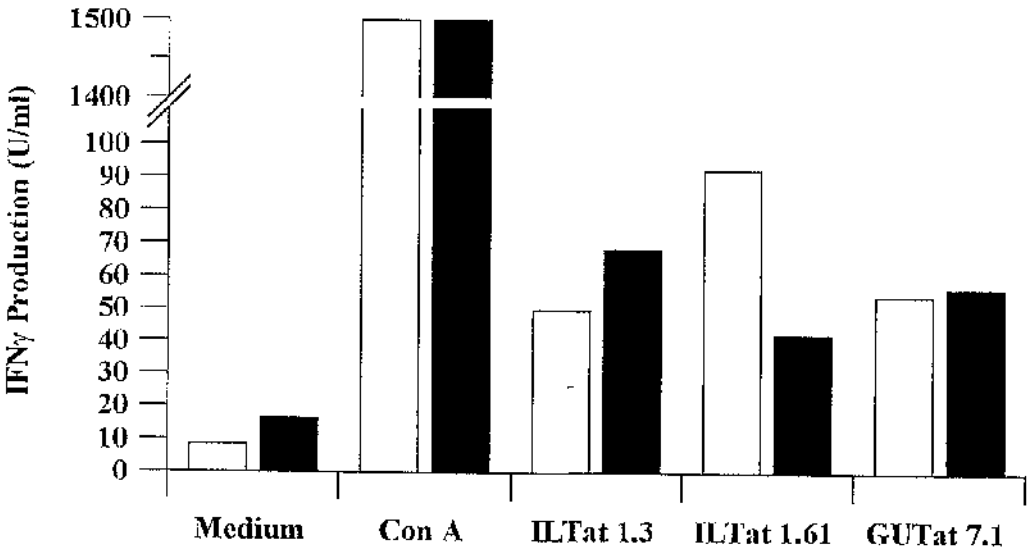


Figure 6.7c. IFNγ production following *in vitro* stimulation of mononuclear splenocytes from mice immunised with peptide 3 then infected with ILTat 1.3.

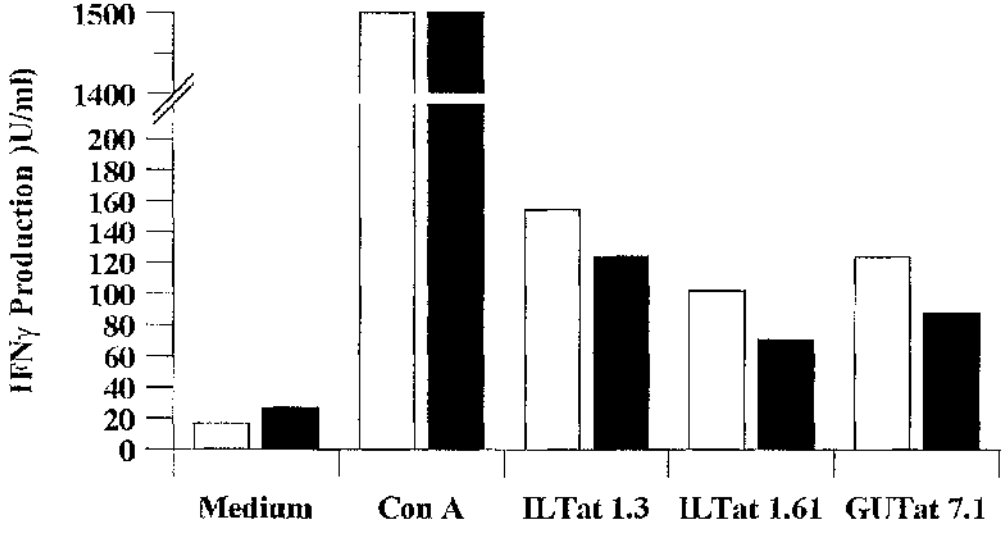


Figure 6.7d. Production of IFNγ by mononuclear splenocytes from mice immunised with peptide 4 and infected with ILTat 1.3 trypanosomes.

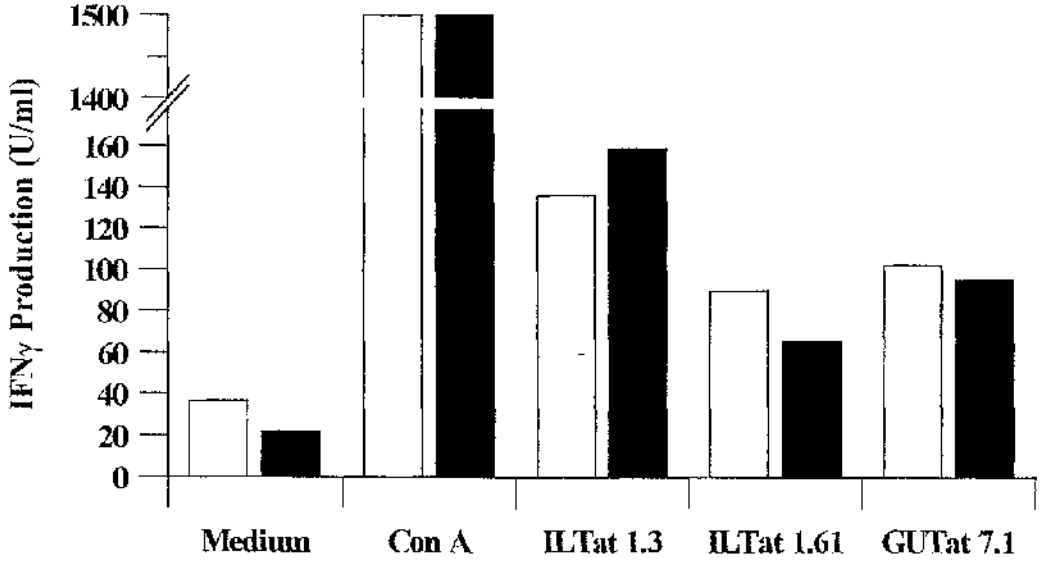


Figure 6.8. IFNγ production was measured from mononuclear splenocytes populations harvested from four groups of mice immunised then infected. The mice were either immunised with peptide 1 (**a**), peptide 2 (**b**), peptide 3 (**c**) or with peptide 4 (**d**) then infected with ILTat 1.61 trypanosomes. The culture supernatants were harvested 24 (white) and 72 (black) hours after *in vitro* stimulation with medium alone, Con A (8μg/ml) and three paraformaldehyde-fixed trypanosome VATs. The results are expressed as the mean values of triplicate wells minus background levels, that is the levels obtained from wells without any sample.

Figure 6.8a. Production of IFNγ by mononuclear splenocytes from mice immunised with peptide 1 and infected with ILTat 1.61 trypanosomes.

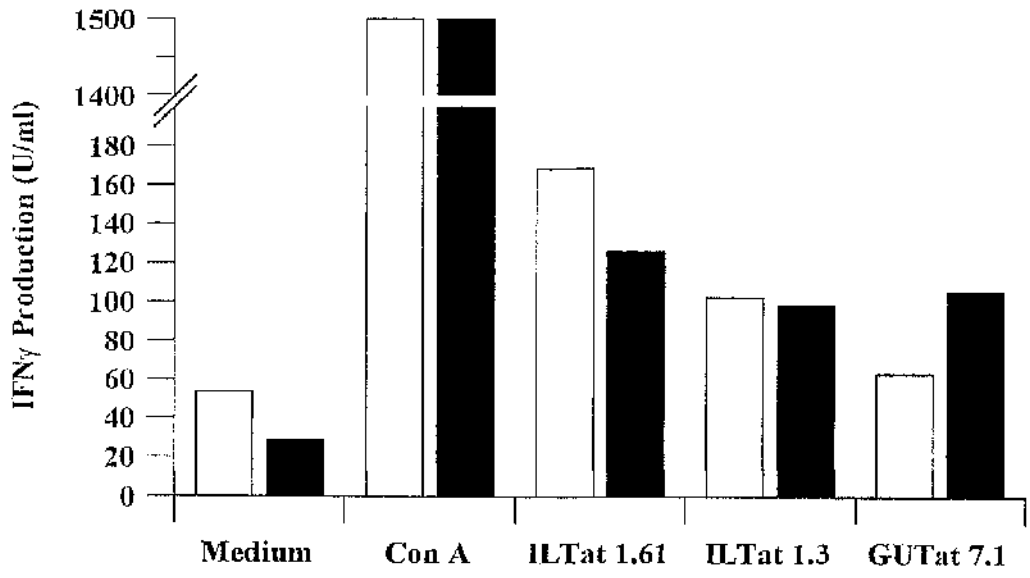


Figure 6.8b. IFNγ production following immunisation with peptide 2 and infection with ILTat 1.61.

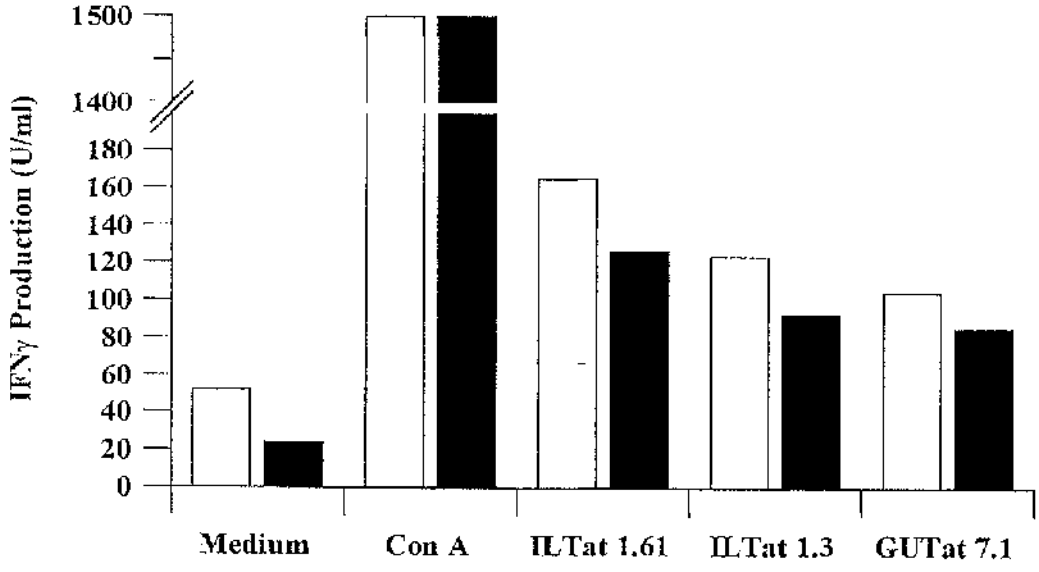


Figure 6.8c. Production of IFNγ from mononuclear splenocytes after immunisations with peptide 3 and infection with ILTat 1.61.

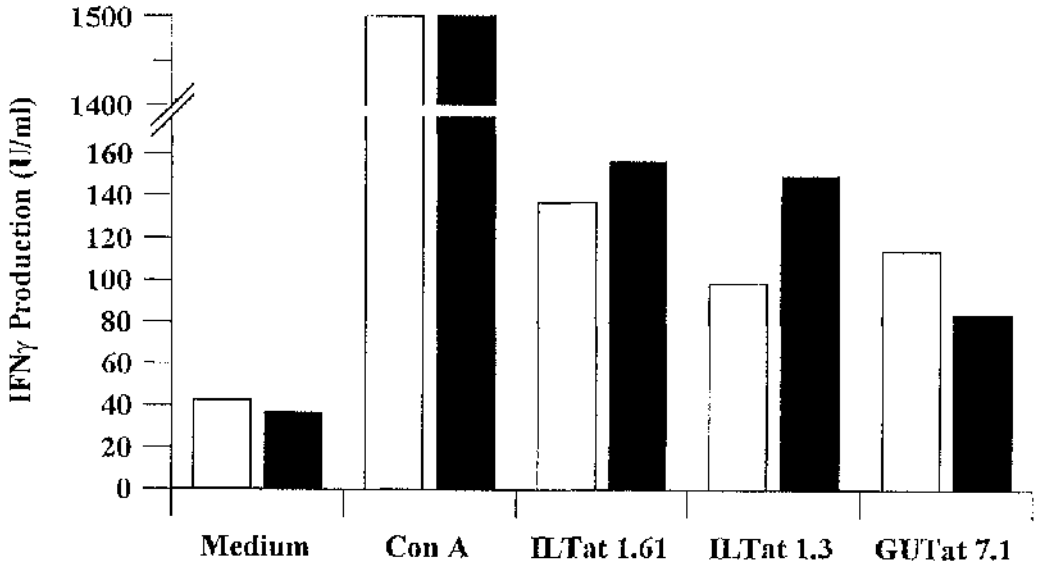


Figure 6.8d. IFNγ production following *in vitro* stimulation of mononuclear splenocytes from mice immunised with peptide 4 then infected with ILTat 1.61.

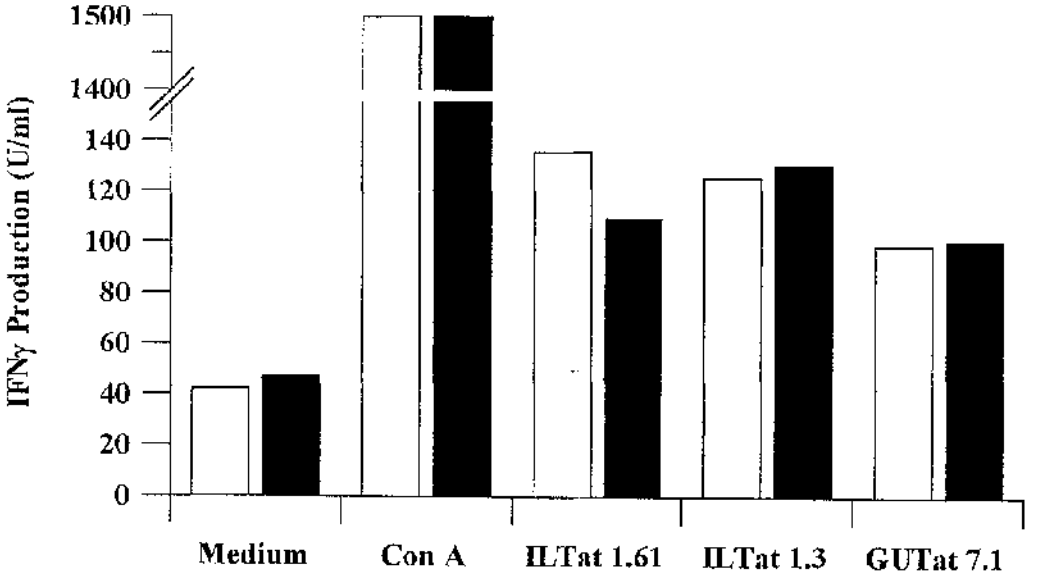


Figure 6.9. The levels of IL-2 production from mitogen-stimulated mononuclear splenocyte populations from mice immunised with one of four peptides, then infected with either ILTat 1.3 (**a**) or ILTat 1.61 (**b**) trypanosomes. The supernatants were examined after 24 (white) and 72 (black) hours and the results are expressed as the geometric means of triplicate wells minus the background levels, that is the levels obtained from the wells which do not contain any sample.

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Figure 6.9a. IL-2 production from mitogen-stimulated mononuclear splenocytes using mice immunised with peptides and then infected with ILTat 1.3.

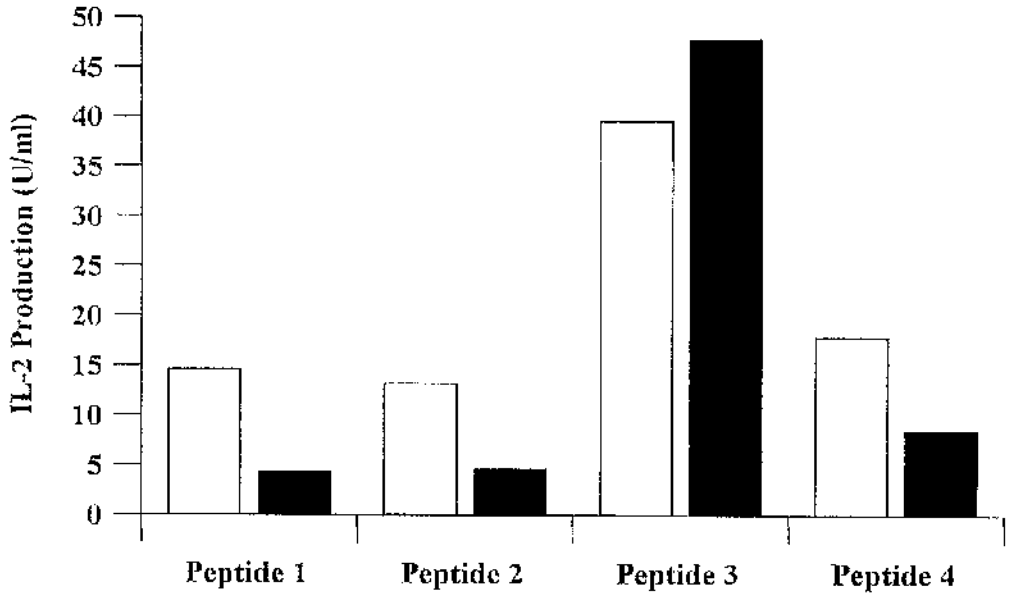
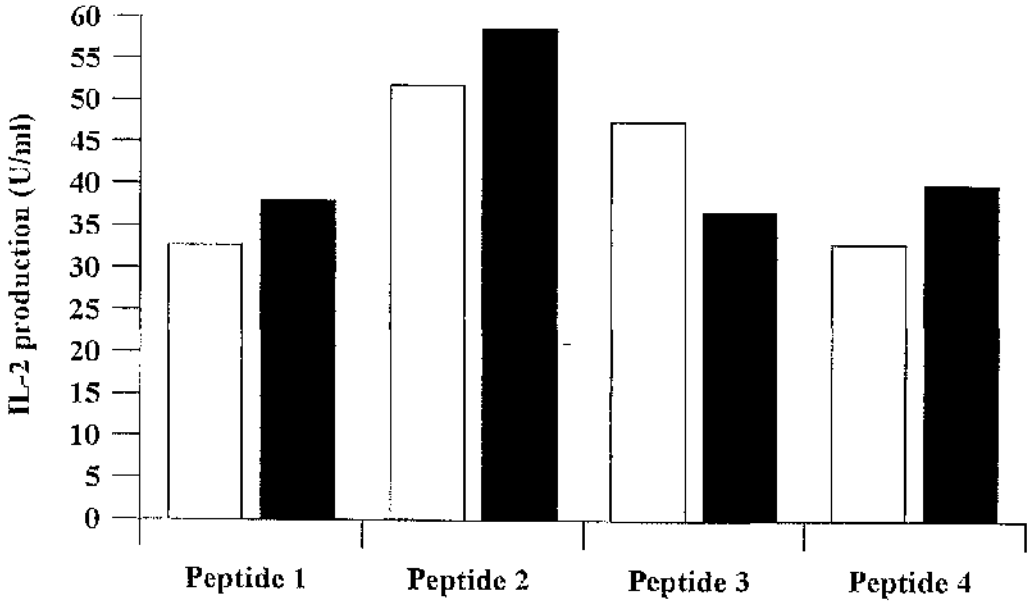


Figure 6.9b. IL-2 production after mitogen stimulation of mononuclear splenocytes following immunisations with peptides and infection with ILTat 1.61.



CHAPTER 7

REGULATION OF T-CELL RESPONSES DURING CHRONIC *T.BRUCEI* INFECTIONS IN MICE LACKING INDUCIBLE NITRIC OXIDE SYNTHASE

7.1. INTRODUCTION

I have presented data in previous chapters which suggests that *T.brucei* parasites can stimulate *ex vivo* T-helper 1 type responses. The question then arises as to how these responses may be regulated. This chapter addresses one potential aspect of this regulation: the role of NO in control of T-cell responses. Two lines of argument suggest, in combination, that NO synthesis may affect T-cell responses and thus the course of a trypanosome infection. A rapid and profound depression of T-cell responses is one of the characteristic features of a trypanosome infection after the first peak of parasitaemia (Corsini *et al*,1977; Jayawardena *et al*,1978; Sileghem *et al*,1986). Macrophages activated during trypanosome infections produce NO and prostaglandins and these macrophages can suppress IL-2 production and IL-2 receptor expression which can in turn affect the T-cell responses (Sileghem *et al*,1989 Sileghem & Flynn,1992; Schleifer & Mansfield,1993). Indomethacin prevents prostaglandin synthesis and indomethacin treatment showed that IL-2 production can be restored after this treatment whereas IL-2 receptor expression cannot (Sileghem *et al*,1989; Sileghem *et al*,1991; Darji *et al*,1993). The inference from these data is that reduced IL-2 receptor expression is influenced by NO. IFNγ appears to play an important role in this putative

regulatory pathway as anti-IFNγ antibodies reduced the level of suppression and restored IL-2 receptor expression (Darji *et al*,1993), at least in some populations.

The second line of argument comes from observation of increased NO synthesis in splenic and peritoneal macrophages from trypanosome-infected mice (Sternberg & McGuigan,1992; Schleifer & Mansfield,1993; Mabbott *et al*,1995). NO has no trypanocidal activity however (Vincendeau *et al*,1992; Mabbott *et al*,1994). Bloodstream trypanosomes are not susceptible to NO-mediated killing *in vivo* as the oxyhaemoglobin present in the RBCs acts as a scavenger for NO (Mabbott *et al*,1994). However, inhibition of NO leads to reduced parasitaemia at the first peak of infection (Mabbott *et al*,1994; Sternberg *et al*,1994) and this may be a result of T-cell functions being at least partially restored.

These arguments can be clearly seen by a summary diagram [Figure 7.1.] which predicts a possible mechanism by which the macrophages induce immunosuppression during *T.brucei* infections: macrophages become activated during infection, produce NO and prostaglandins which in turn depress IL-2 receptor expression and IL-2 production respectively. Both events result in a depressed T-cell response thereby allowing an increase in parasitaemia.

According to this line of reasoning, NO inhibition reduces trypanosome parasitaemias by releasing the T-cells from their depressed state, which is mediated by the downregulation of IL-2 receptor expression, and thereby restoring T-cell competence. The prediction would be therefore that abrogation of iNOS activity to prevent a rise in NO levels in infections should lead to upregulation of indicators of T-cell activity and improved control of parasitaemia. I therefore investigated the possibility that NO synthesis may affect the course of a trypanosome infection via control of T-cell responses using mice lacking iNOS (Wei *et al*,1996).

7.2. RESULTS

7.2.1. Comparison Of Parasitaemia In iNOS-deficient And Control Mice

During infection with the pleomorphic GUTat 7.2 trypanosomes, the parasitaemias in iNOS-deficient and control mice progressed at similar rates towards the first peak of parasitaemia [Figure 7.2.]. Both groups reached first peak of parasitaemia at the same day post-infection and essentially the same levels of parasitaemia before parasitaemias declined with the onset of effector immune responses. In the iNOS-deficient group, the parasitaemias decreased at a quicker rate during the chronic phase of the infection when compared to their heterozygous counterparts in which parasitaemias remained at a higher level for a longer period of time before declining. From days 8-11 of infection, there was an approximate order of magnitude difference in levels of parasitaemia between iNOS-deficient and control mice.

Interestingly, the iNOS-deficient mice appeared clinically more distressed by infection than the control mice. They were more sluggish, had badly piled coats, limp tails, dull eyes and their breathing was more shallow even though these mice had fewer parasites in their bloodstream than the control mice. Because of these changes in clinical presentation the mice had to be killed on day 11 of infection.

7.2.2. Immunoglobulin Isotyping Of Plasma Samples

Immunoglobulin isotype levels were determined for the uninfected and infected iNOS-deficient mice and their heterozygous counterparts [Figure 7.3.]. Comparing the

isotype levels of the uninfected groups with each other and of the infected groups with each other, the levels were fairly similar in all instances. These data suggest that the B-cells in the homozygous and heterozygous mice, as indexed by immunoglobulin titre, are not affected if inducible nitric oxide synthase is lacking. In the control trypanosome-infected mice, where iNOS production was intact, the B-cells showed no indication of being immunodepressed as far as their immunoglobulin production was concerned for the isotypes examined.

At day 11 post-infection, it was shown that the level of IgM had increased several fold compared to the IgM levels of the uninfected mice. The levels of IgG_{2a} increased by approximately 200mg/litre in the infected groups with the IgG_{2b} isotype levels also increasing slightly above those of the uninfected mice. However, the levels of the IgG_1 immunoglobulins decreased by approximately 200mg/litre for both infected homozygous and heterozygous mice.

7.2.3. Proliferative T-cell Responses

When the ability of the T-cells to respond, in a proliferative manner, to medium or Con A was examined it could be clearly shown that the degree of response differed between the trypanosome-infected controls and the infected iNOS-deficient mice [Figure 7.4.]. The proliferative T-cell response, after Con A stimulation, in the iNOS-deficient mice was approximated double that of the heterozygous infected mice.

When the mononuclear splenocytes from infected groups of mice were *in vitro* stimulated with medium alone, the cells from the iNOS-deficient mice responded with high ^{3}H-Thymidine incorporation values compared with the heterozygous counterparts. The high levels of proliferation in both groups stimulated with medium is probably due to

the cells being highly primed *in vivo*, due to the trypanosomes, and therefore when transferred to the *in vitro* environment the cells will carry over with them partially processed antigen. These high values have also been observed in infected Balb/c mice (Chapters 3 & 4). The higher levels of proliferation in iNOS-deficient compared with control mice may result because the homozygous mice are not under iNOS-induced immunodepression, they therefore produce a greater T-cell proliferative response than the control mice with regards to *in vitro* stimulation both with Con A and with medium alone.

When the values, provided by ^{3}H-Thymidine incorporation, were examined to provide a stimulation index for each Con A response, the responses differed between experiments. In Figure 7.4., the stimulation index for the homozygous mice was 4.5 whereas it was 2.9 for the heterozygous mice. However, in a repeat experiment (results not shown) the stimulation indices were almost identical with the homozygous mice producing a stimulation index of 3.3 and the heterozygous controls producing an index of 3.2.

7.2.4. Cytokine Production In Control And iNOS-deficient Infected Mice

Splenocytes from chronically infected mice were different in their capacity to produce IFNγ when compared between the control and iNOS-deficient mice [Figure 7.5.]. The GUTat 7.2, ILTat 1.3 and ILTat 1.61 paraformaldehyde-fixed trypanosomes, which were used for *in vitro* stimulation, are all expressed at some point during the GUTat 7.2 chronic infection with GUTat 7.2 being the 'homotype' at the first parasitaemic peak. Mononuclear splenocytes from the control mice produced very little IFNγ when stimulated with any one of the three paraformaldehyde-fixed VATs and very low

amounts was detected even when stimulated with Con A. However, IFNγ production with the mononuclear splenocytes from the iNOS-deficient mice was very different. Very little IFNγ was detected in the samples stimulated with medium alone, as with the control mice, and only slightly more than this with the ILTat 1.61-stimulated cells. The ILTat 1.3-stimulated cells produced several fold more IFNγ than the ILTat 1.61-stimulated cells but not nearly as much as those cells stimulated with GUTat 7.2 trypanosomes. The cells stimulated with Con A produced the highest IFNγ response. The IFNγ production was 2-fold greater than the GUTat 7.2 response and above 1000U/ml.

The levels of IL-5 and IL-2 production was also ascertained. In both the control and iNOS-deficient infected groups, there was no IL-5 production above 1U/ml when stimulated with medium, Con A or one of the three fixed VATs (data not shown). When the levels of IL-2 were measured, the only detectable IL-2 production was from the cells stimulated with Con A. These levels, in both groups of mice, were less than 10U/ml 24 hours after stimulation *in vitro* (data not shown).

7.2.5. FACS Analyses Of Mononuclear Splenocytes

When the numbers of CD4 and CD8 T-cells were counted for each spleen, the mean values for the uninfected mice showed that there were approximately the same numbers of both $CD4^+$ and $CD8^+$ cells present in the spleens from the control group compared with the iNOS-deficient group [Figure 7.6.]. By day 11 of infection, the CD4 population from the iNOS-deficient mice had increased dramatically compared with its uninfected counterpart whereas there was no significant increase in numbers in the heterozygous mice. In the case of the CD8 T-cell populations, there was a very slight decrease in the

number of $CD8^+$ cells/spleen in the infected mice when compared to the uninfected groups both for iNOS-deficient and control mice.

The percentages of CD4 and CD8 T-cells in the mononuclear splenocyte populations were calculated as well as the CD4:8 ratio and the ratios of activated T-cell sub-populations [Table 7.1.]. The percentage abundances of CD4 and CD8 cells in uninfected mice were lower in heterozygous than in homozygous mice implying that, since the numbers of each cell type/spleen were similar, the spleens in iNOS-deficient mice might be of a different architecture compared with control mice. The percentage of CD4 cells increased in the infected iNOS-deficient mice whereas they decreased in the control group. However the percentage of CD8 cells decreased in both the control and iNOS-deficient mice when chronically infected compared with their uninfected counterparts. Despite these different changes in percentages of CD4 and CD8 cells with infection, the CD4:CD8 ratios of the uninfected groups was almost identical and when the infected groups were compared, the 8:1 ratio of the iNOS-deficient group did not differ dramatically from the heterozygous group, which had a ratio of 7.3:1.

The ratios of activated CD4 cells examined using CD25 as a marker of activation were informative [Table 7.1.]. There were 3-fold more activated CD4 cells present in the spleens from iNOS-deficient infected mice compared with those of spleen from infected controls. There were no detectable activated CD8 cells in the mononuclear splenocyte populations from the uninfected control and iNOS-deficient mice or from the infected control mice but a small number were present in the infected iNOS-deficient mice.

7.2.6. Splenic Architecture

The Haematoxylin and Eosin stained spleen sections were examined at low magnification, ×10, from the uninfected heterozygous [Figure 7.7a.] and homozygous [Figure 7.7c.] mice. The splenic architecture appears intact and highly structured in the uninfected control and iNOS-deficient mice with distinct red and white pulp areas and lymphoid follicles in both mice. Examination of the spleen sections from the trypanosome-infected control [Figure 7.7b.] and iNOS-deficient [Figure 7.7d.] mice showed a lack of splenic structure with no distinction between red pulp and white pulp areas in the spleens from either of these mice. These observations can be explained by the trypanosome infection causing an influx of cells into the spleen which invades the stromal areas resulting in a loss of architecture. There was no apparent differences between those spleens from the homozyogus and heterozygous mice. At a higher magnification, ×40, the section from the uninfected control mouse showed a lymphoid follicle surrounded by a marginal zone with the cells of a uniform size [Figure 7.7e.] but when compared with the cells from an infected control mouse, at the same magnification, it was shown that there was no structured follicle, the cells were larger in size and there was no noticeable stromal region
[Figure 7.7f.].

7.2.7. Plasma Nitrate

Using the Greiss Reaction to measure the nitrate levels in plasma samples, the background levels of nitrate production were 20-30μM concentration in both control and iNOS-deficient mice uninfected mice [Figure 7.8.]. In the infected control mice there was a significant increase in plasma nitrate levels, above 160μM, and, slightly

surprisingly, an increase was also observed in the nitrate levels in the iNOS-deficient mice which increased more than 2-fold in chronically infected mice.

7.3. DISCUSSION

The possibility that NO synthesis may affect the course of a trypanosome infection via control of T-cell responses was investigated using iNOS-deficient mice. Daily monitoring of infections showed that the parasitaemia increased at the same rate towards the first peak of parasitaemia in both the iNOS-deficient and control mice and that the peak parasitaemia levels were the same in both groups. However, the control mice maintained a higher parasitaemia in the chronic phase of an infection than the iNOS-deficient mice due to a decrease in the rate of clearance of parasites. These data contrast with the results of Sternberg *et al* (1994) where mice were shown to have 50% reduced peak parasitaemias when given L-NAME in their drinking water but a few days after this there was no significant difference in the parasitaemia of these mice compared with the mice given water or D-NAME, a substrate analog inhibitor of NOS which is biologically inactive (Sternberg *et al*,1994). It is difficult to offer an explanation as to why parasitaemias in iNOS-deficient and L-NAME treated mice should be so different but it may be worth noting that in a separate study Houston & Turner (unpublished results) were unable to replicate the results of Sternberg *et al* (1994). Alternatively, it is possible that the difference lies simply in the use of different strains of both trypanosomes and mice in the two studies. *T.brucei* trypanosome growth was prevented *in vitro* when exposed to activated macrophages but this inhibition was removed when L-NMMA (Vincendeau *et al*,1992) was added, indicating that NO was having a direct cytostatic effect in the parasites. However, dilutions of whole blood (Mabbott *et al*,1994) also

removed growth inhibition, presumably by quenching of NO by oxyhaemaglobin and suggesting that bloodstream trypanosomes *in vivo* will not be directly susceptible to killing or cytostasis by NO. Further support for this notion comes from the observation that SNAP, a NO donor, reduced trypanosome proliferation *in vitro* by 75% (Sternberg *et al*,1994) but this effect was removed by the addition of whole mouse blood. Therefore the action of NO is likely to be indirect by modifying effector immune responses.

The infected control mice looked healthier for a longer period of time compared to the iNOS-deficient mice. One explanation for this result may be an immense decrease in the numbers of trypanosomes present in the iNOS-deficient mice, due to the greater immunocompetence of the immune system, leading to an increase in the amount of parasite antigen released and immune complex formation. Immune complex formation is thought to underlie much of trypanosome-induced pathology (Jenkins & Facer,1984). The formation of immune complexes and release of soluble parasite antigens may be more gradual occurring over a longer period of time due to the depression inflicted on the host cells.

Titres of immunoglobulin isotypes were similar between the two infected groups and also similar between the uninfected groups, suggesting that the reduction in iNOS does not dramatically affect the amount of immunoglobulin produced by the B-cells. It also indicates that although the infected control mice were immunosuppressed, this suppression did not detectably suppress the B-cells in their ability to produced immunoglobulins. Suppressed antibody responses have however been detected in other studies. Complete suppression of IgM and IgG responses to sheep RBCs was observed during acute and sub-acute trypanosome infections in mice (Sacks *et al*,1980). In chronic infections however, the IgG response was completely suppressed but the IgM

response only partially suppressed. The important reservation with this study is that suppression was against non-parasite antigens rather than suppression of the response against the trypansomes themselves. Trypanosome specific antibody responses have been shown to be suppressed during infection by Sacks & Askonas (1980). Normal mice, immunised with irradiated trypanosomes, had far greater total immunoglobulin levels to the irradiated trypanosomes than the chronically or acutely infected mice which were immunised with irradiated trypanosomes, of a different antigen type, with the acutely infected mice having the lowest antibody titres of the three groups (Sacks & Askonas,1980). During the chronic infection, the IgG and IgM titres to homologous trypanosomes decreased with each successive wave of parasitaemia with the IgG titres declining more rapidly than the IgM (Sacks & Askonas,1980). The sera were taken from the mice 10 days after a parasitaemic peak and the specific IgM and IgG titres determined but it could be that these specific isotypes produced a good specific response which was complete and had disipated by day 10 when the samples were taken. It may be possible that my mice were still too early in their chronic phase of infection to show a suppression in immunoglobulin isotypes.

The T-cell proliferative responses were greater in the mice where iNOS was lacking compared with the control mice and this was seen *in vitro* both with Con A and in the absence of any. During chronic infections, the immune system is continually fighting the infection and this may result in clonal exhaustion and therefore a decreased proliferative response. The presence of NO may contribute to depressing this response. Mabbott *et al* (1995) have shown that when the NO synthesis from macrophages was inhibited, either *in vivo* or *in vitro*, the suppression of proliferation was partly revoked. They showed suppression of splenocyte proliferation, stimulating with Con A, when macrophages from infected mice were introduced to cultures from naive mice. The

addition of L-NAME or L-NMMA partially abrogated this suppression and this was observed both *in vitro* and *in vivo* (Sternberg & McGuigan,1992; Schleifer & Mansfield,1993; Sternberg *et al*,1994; Mabbott *et al*,1995) suggesting the involvement of both NO and another mediator of suppression, probably prostaglandins. The fact that the lack of the proliferative response can be overcome suggests that clonal exhaustion may be the cause of the lack of T-cell proliferation observed in the infected control mice.

The prostaglandin E_2 levels which accumulated in cultures were not significantly altered by blocking NO production with L-NMMA but prostaglandin E_2 production was blocked by indomethacin treatment (Schleifer & Mansfield,1993). However inhibiting prostaglandin synthesis alone did not reverse the suppressed T-cell proliferation. Levels of proliferative T-cell responses were completely restored in co-cultures of cells, from infected and uninfected mice, when L-NMMA and indomethacin were added simultaneously (Schleifer & Mansfield,1993). This evidence that abrogation of NO leads to, at least partial, recovery of responses suggests that suppression is related to clonal exhaustion in the classic sense in that the cells have not been clonally deleted but have reversible inability to respond to an antigen. Prostaglandins therefore play a role, albeit a small role compared to that played by NO, in the suppression of the immune response in trypanosome-infected mice.

In vitro experiments showed that the addition of splenocytes, up to 10% of the total, from infected mice to cutures of splenocytes from naive mice resulted in up to 50% suppression of the T-cell proliferative response when stimulated with Con A whereas 100% splenoctyes from trypanosome-infected mice resulted in more than 90% suppression with mitogen stimulation (Sternberg & McGuigan,1992). However L-NAME could partially remove this suppression. Sileghem *et al* (1989) revealed that a T-cell enriched fraction of cells from infected mice was not suppressive. They examined

the responsiveness of lymph node cells and showed that the cells responsible for the suppression during trypanosome infections were those which reduced IL-2 receptor expression. The cells which are responsible were purified in the macrophage fraction of cells, were nylon wool adherent, plastic adherent, fibronectin R^+, Mac-1^+ and Thy-1^-. It was also shown that cells which fail to express Mac-1, that is cells which are not macrophages, cannot suppress the responsiveness of lymph node cells from uninfected mice (Sileghem *et al*,1989).

It could have been that there was a degree of depletion of T-cells in infected control mice which led to the reduced proliferative and cytokine responses. However analyses, by flow cytometry, have shown that although the percentage of CD4 and CD8 T-cells decreased in the infected control mice the CD4 cell numbers/spleen increased. Unfortunately, it is not obvious as to which is the more important criterion, the number of CD4 cells/spleen or the relative proportions of cell types. I have shown that the number of activated cells was significantly reduced in the control mice compared to the iNOS-deficient mice and this could be seen in both the CD4 and CD8 T-cell populations (particularly the former) and although IL-2 has a central role in T-cell activation and proliferation there was no detectable IL-2 when the samples from the infected control and iNOS-deficient mice were examined by ELISA. It has been shown previously that during chronic murine infections it is not the depletion of T-cells or the absence of functional accessory cells which causes the impaired T-cell proliferative responses in the lymph nodes but the lack of, or suppression of IL-2 production (Sileghem *et al*,1986).

The addition of recombinant IL-2 to cells from infected and uninfected mice revealed that the cells from the infected mice required a longer exposure to the IL-2 in order to reach their optimum proliferative potential compared with the cells from the uninfected mice (Sileghem *et al*,1986). This could possibly be explained by the downregulation of

IL-2 receptor expression. The resulting recovery of T-cell proliferation, by an exogenous source of IL-2, suggests that these cells from trypanosome-infected mice are anergic. Both IL-2 and IL-2 receptor expression are suppressed by macrophages from infected mice (Sileghem *et al*, 1991). A prostaglandin-dependent mechanism is responsible for the suppression of IL-2 whereas IL-2 receptor expression is suppressed by a prostaglandin-independent mechanism as shown by indomethacin treatment which allows IL-2 secretion to occur but does not restore IL-2 receptor expression (Sileghem *et al*, 1991). This suggests that there are two mediators of suppression acting, that is prostaglandins, suppressing IL-2 production, and NO, suppressing IL-2 receptor expression.

Very low levels of IFNγ were produced by the mononuclear splenocyte populations from the control mice which contrasts with the high levels of IFNγ production by the isolated cells from the iNOS-deficient mice. It could be suggested that IFNγ is an important factor in the immune response towards the first parasitaemic peak but in the chronic phase of infection the IFNγ which has been produced stimulates the host macrophages resulting in immunosuppression. Therefore in the infected control mice there is a very depressed IFNγ response. IFNγ may be a 'double-edged sword' in that it benefits the host, by stimulating a T_H1 type response, during the acute phase of infections but during the chronic phase it aids the parasite by assisting in the immunosuppressive effects caused by the activated macrophages. An increase in IFNγ production was observed when L-NMMA was included in cell cultures (Schleifer & Mansfield, 1993) which concurs with my data in that the iNOS-deficient mice have significantly higher levels of IFNγ compared with the infected control mice. It was recently revealed that a NOS-activating factor is present in bloodstream *T. brucei* parasites (Sternberg & Mabbott, 1996) and in the presence of IFNγ becomes active. It was previously reported

that lymph node cells from *T.brucei*-infected mice could produce IFNγ even though IL-2 production was suppressed (Sileghem *et al*,1986) which contrasted with what I observed using mononuclear splenocytes from infected mice.

Darji *et al* (1993) highlighted the importance of IFNγ in the suppression of the host immune response during trypanosome infections. Anti-IFNγ antibodies were added to co-cultures of lymph node cells from infected and uninfected mice and the level of IL-2 receptor expression was restored and the degree of suppression reduced resulting in the proliferation of these cultures to normal levels (Darji *et al*,1993). This group showed that anti-IFNγ antibody treatment decreased parasitaemia by 50% during first peak but after this period the parasitaemia was the same as the untreated mice and no difference in survival times between the groups was observed. Darji *et al* (1993) also showed that anti-IFNγ antibody treatment six days post-infection restored Con A-induced proliferative responses in the lymph node cells of infected mice but the spleen cells were unaffected by this treatment suggesting lymph node cells but not spleen cells need IFNγ to induce unresponsiveness *in vivo*. The biological relevance of data that pertains only to lymph node cells for a bloodstream infection must remain questionable. Anti-IFNγ monoclonal antibodies removed the increased NO production in spleen cell cultures stimulated with mitogen or antigen and also reduced the basal levels of NO present (Schleifer & Mansfield,1993). Darji *et al* (1993) proposed that as well as the important pivotal role in activating macrophages, thereby generating immunosuppressive consequences, IFNγ is directly involved in the suppression of T-cell proliferation and depressed IL-2 receptor expression in the lymph nodes. Again, this contrasts with my data for the spleen which shows that high levels of IFNγ are produced by the iNOS-deficient mice, and although IL-2 was not detected, there were still greater levels of IL-2 receptor expression on the CD4 and CD8 cells compared with the levels in the control

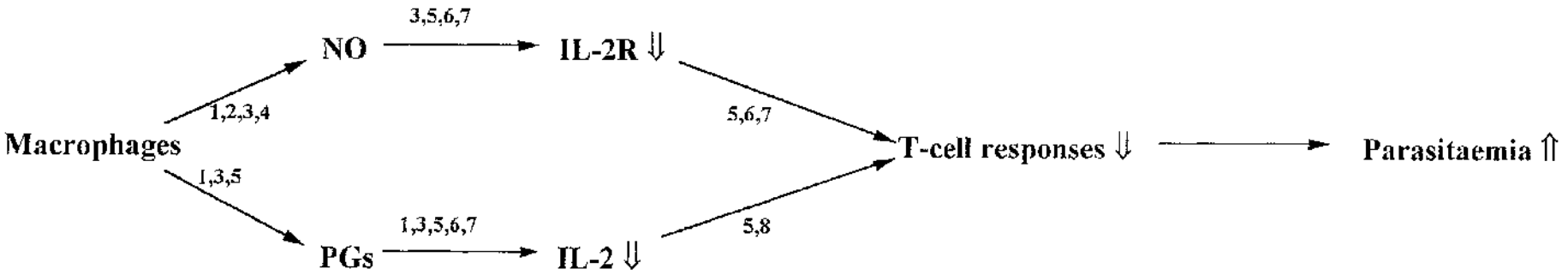
mice. Instead, it appears more likely that the anti-IFNγ antibodies neutralise the IFNγ which means less macrophage activation and therefore less NO production allowing increased proliferation and IL-2 receptor expression.

Plasma nitrate levels were several fold greater in the infected control mice compared to the uninfected mice showing background levels as would be expected from previous observations (Schleifer & Mansfield,1993; Kantor *et al*,1996; Sternberg & Mabbott,1996; Taylor *et al*,1996). There was, however, an increase in the nitrate levels in the infected iNOS-deficient mice compared with uninfected mice. This may be because the iNOS-deficient mice are slightly 'leaky' or because another enzyme can upregulate its synthesis of NO production to compensate for the lack of iNOS activity. Residual endothelial NOS activity has previously been detected in neuronal NOS knockout mice and endothelial NOS can compensate for the loss of neuronal NOS to allow functions to continue (Kantor *et al*,1996). After neuronal injury or toxic insults to cells, the cells increase their NOS protein levels (Samdani *et al*,1997). It is therefore conceivable that during trypanosome infections, the large amount of anitgen and alterations in splenic architecture results in cell injury and vascular disruption producing an increase in NO production from the other distinct NOS genes.

At the present time it is not possible to distinguish categorically the individual components of the immune response and the importance each possesses, either as a positive or a negative regulator in the immunosuppression during African trypanosome infections due to the complex immune network of the host. It is clear however that macrophages are the crucial cell type involved in causing immunosuppression during these infections by way of NO and prostaglandin production. I would suggest that when the trypanosomes infect their host, they stimulate an IFNγ response which will activate the macrophages. These activated macrophages will in turn produce NO and

prostaglandins to induce suppression of the immune response. The NO acts by downregulating IL-2 receptor expression whereas the prostaglandins suppress IL-2 production. These changes in turn cause depression of T-cell, but not B-cell, responses resulting indirectly in an increase in parasitaemia. By removing NO, dramatic differences were observed in parasitaemia, T-cell proliferation, cytokine production and IL-2 receptor expression.

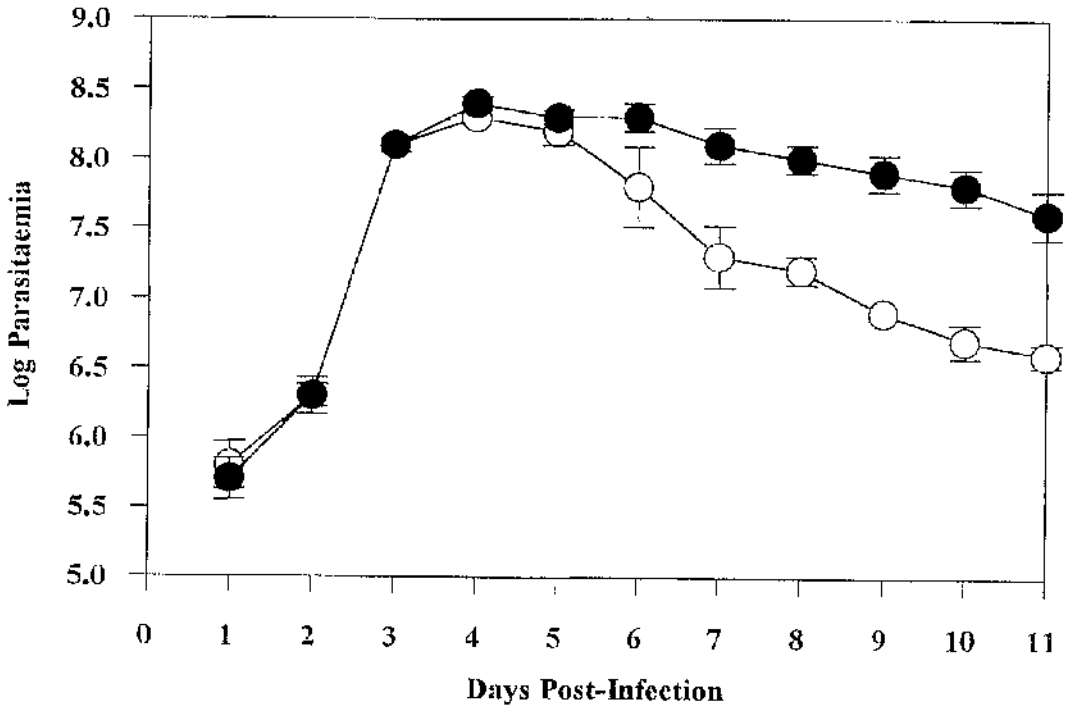
Figure 7.1. Suggested mechanism of macrophage-induced immunodepression during chronic *Trypanosoma brucei* infections.



The current literature shows that activated macrophages produce nitric oxide (NO) and prostaglandins (PGs) during *Trypanosoma brucei* infections which act by downregulating (⇓) IL-2 receptor (IL-2R) expression and IL-2 production respectively. These alterations to the immune response will have a downstream affect on host T-cell responses and ultimately on the parasitaemia.

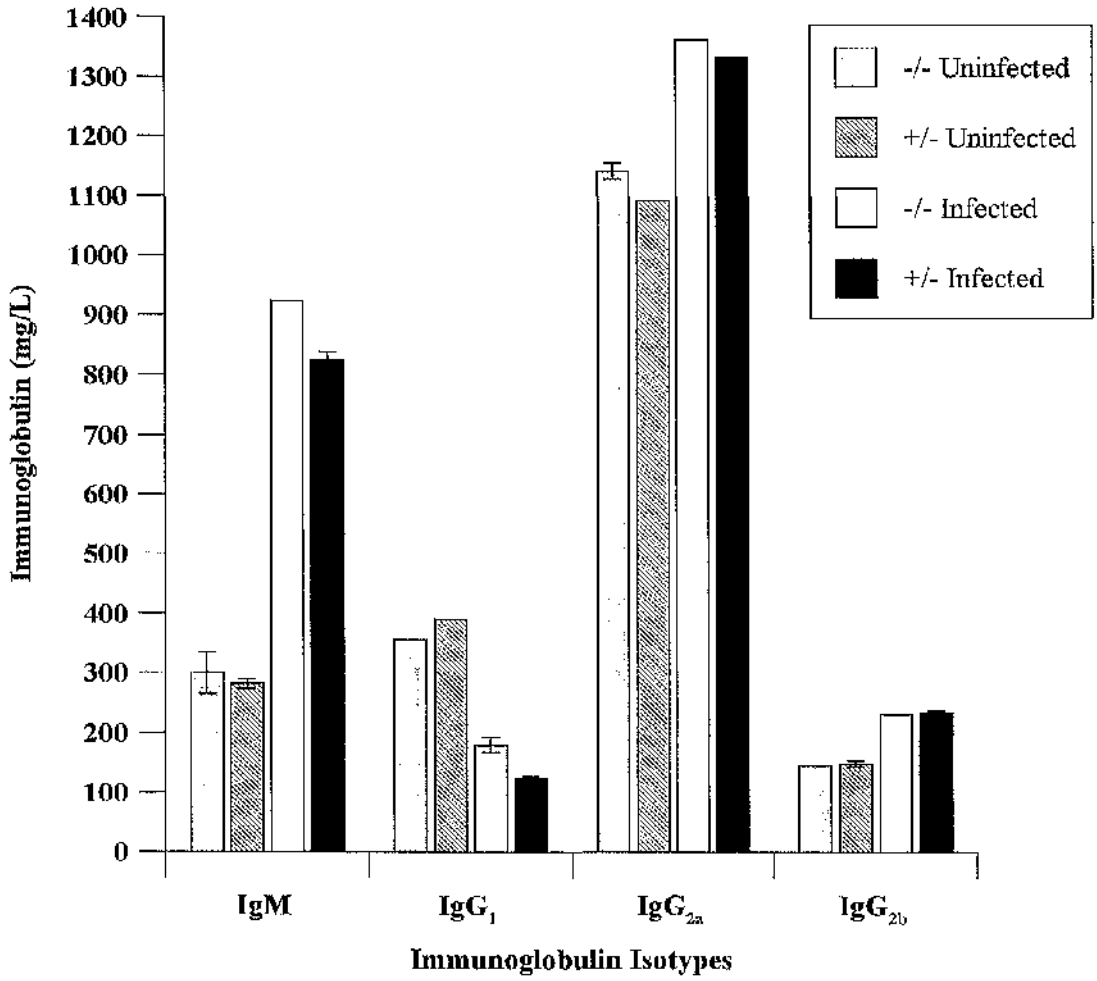
1. Sileghem *et* al,1991
2. Sternberg & McGuigan,1992
3. Schleifer & Mansfield,1993
4. Mabbott *et* al,1995
5. Sileghem *et* al,1989
6. Sileghem & Flynn,1992
7. Darji *et* al,1993
8. Sileghem *et al*,1986

Figure 7.2. Parasitaemia of GUTat 7.2.



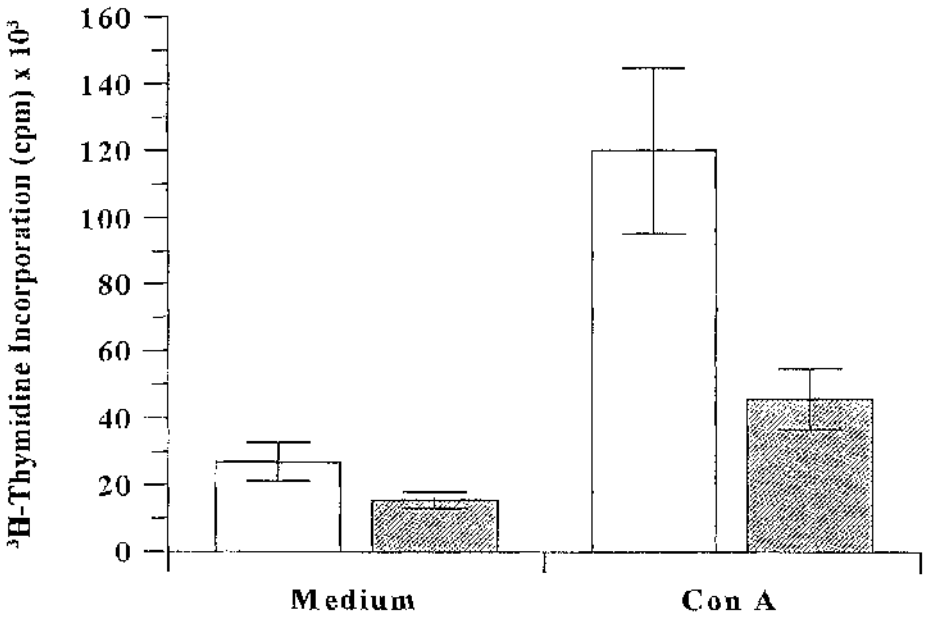
The parasitaemia of iNOS-deficient (○) and control (●) mice was determined daily by the removal of blood samples from each mouse, diluted in appropriate concentrations of ammonium chloride and counted on an Improved Neubauer haemocytometer. Results are expressed as geometric means ± 2 SE, n=4.

Figure 7.3. Immunoglobulin isotyping for control and iNOS-deficient mice.



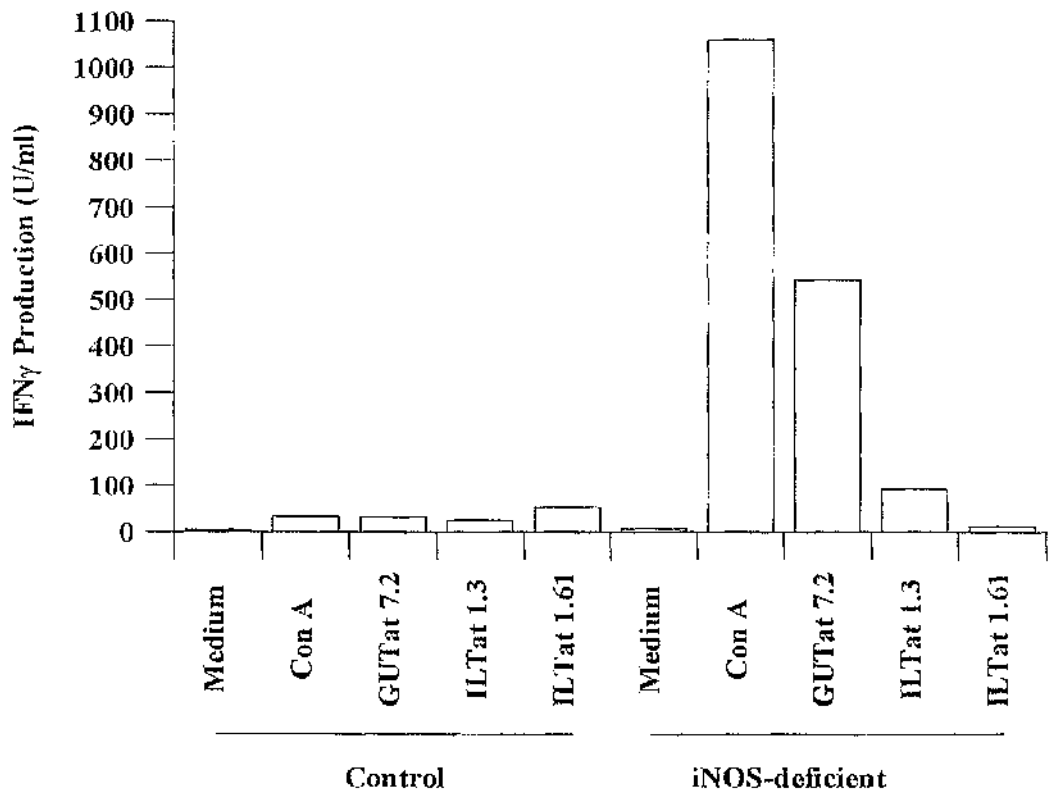
Immunoglobulin isotypes were measured by radioimmunodiffusion using the plasma samples from individual mice infected with GUTat 7.2 trypanosomes on day 11 of infection and uninfected control mice. Mice were either homozygous and deficient (-/-) for the iNOS locus or heterozygous (+/-) for that locus. The results are expressed as the geometric mean ± 2 SE, n=4. Some sample groups do not display error bars and this is because they had standard errors of zero.

Figure 7.4. Proliferative responses of mononuclear splenocytes from mice on day 11 of infection with GUTat 7.2 trypanosomes.



Medium alone and Con A were used to stimulate cells and proliferative responses assayed by ^{3}H-Thymidine incorporation. Responses were compared in cells from mice lacking iNOS (white bars) and heterozygous control (hatched bars) mice. The results are expressed as the geometric mean ± 2 SE, n=4.

Figure 7.5. IFNγ production by mononuclear splenocytes from mice chronically infected with GUTat 7.2.



Cells from infected control (+/-) and iNOS-deficient (-/-) mice were stimulated *in vitro* with medium alone, Con A or paraformaldehyde-fixed trypanosomes expressing one of three VATs. Culture supernatants were examined by sandwich ELISA and the mean level of IFNγ produced determined from triplicate assays.

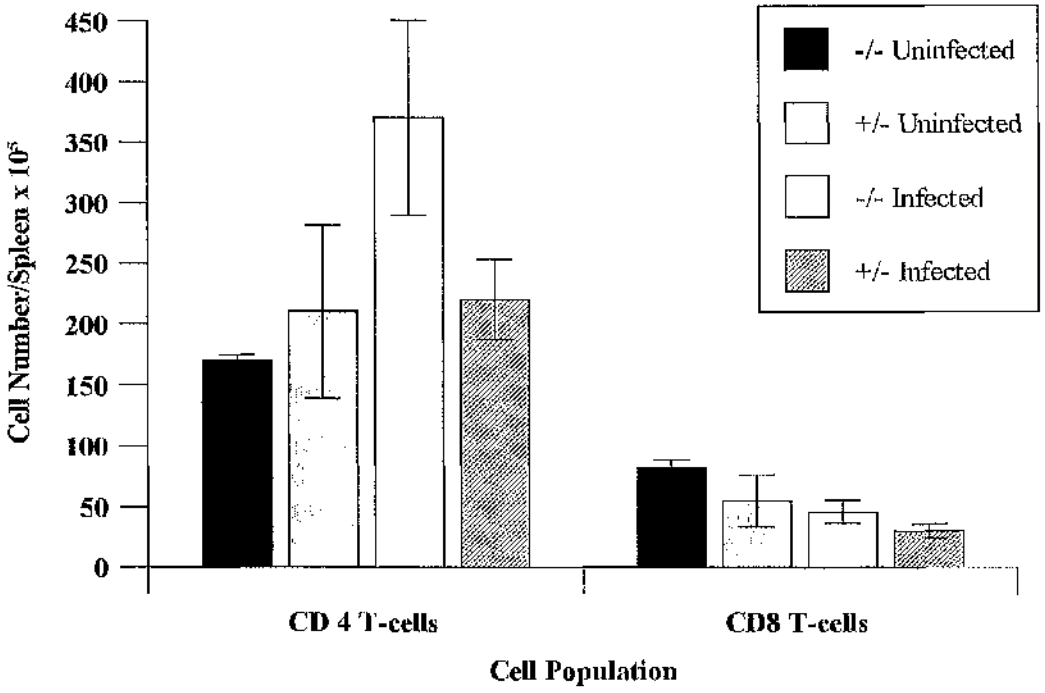
Table 7.1. The effects of trypanosome infection on percentages of CD4 and CD8 cells, together with ratios of CD4:8 and of activated CD4 and CD8 cells in mononuclear splenocytes of mice either homozygous and lacking (-/-) the iNOS locus or in heterozygous (+/-) controls.

Cell Type	-/- Control	+/- Control	-/- Infected	+/- Infected
CD4 T-cells	24.3 ±2.0	17.0 ±1.4	34.0 ±8.9	12.7 ±1.5
CD8 T-cells	11.3 ±1.5	7.5 ±0.7	4.3 ±1.1	1.7 ±0.6
CD4:CD8 ratio	2:1	2.3:1	8:1	7.3:1
CD4:CD25 ratio	100:1	96:1	47:1	157:1
CD8:CD25 ratio	ND	ND	229:1	ND

Percentages and ratios were determined from FACS analyses of cells from invividual mice, results pooled from four mice and expressed as mean values ± 2 SE, n=5000. Mice were either uninfected or on day 11 of a GUTat 7.2 trypanosome infection. CD25 was employed as the activation marker.

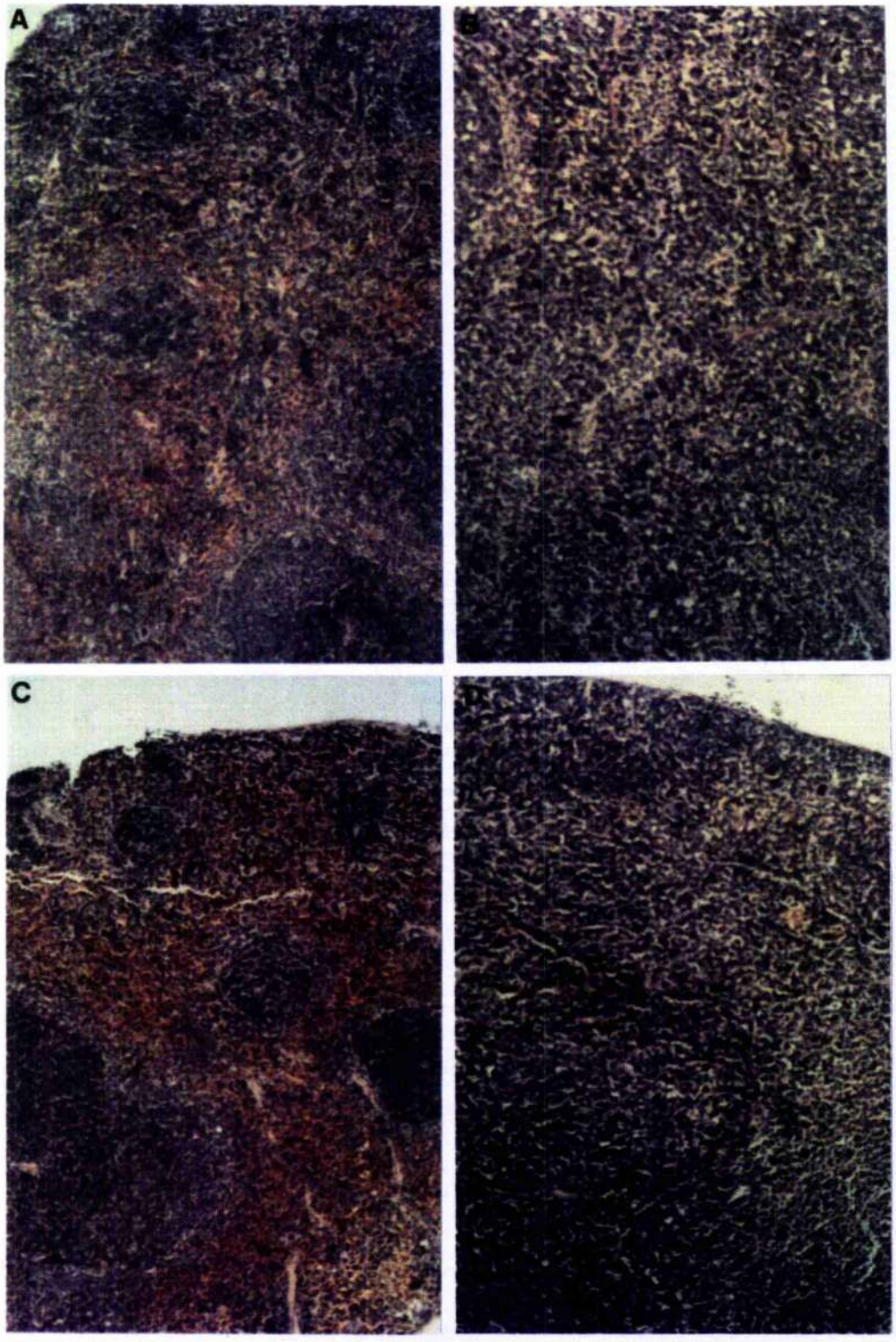
ND = activated CD8 cells were not detectable.

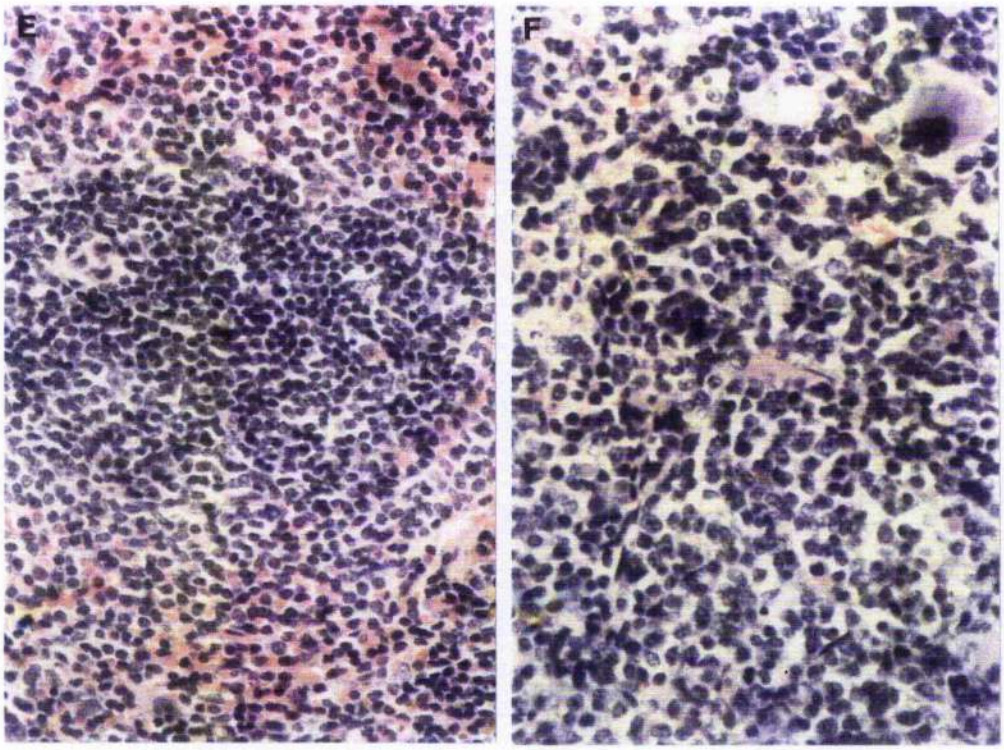
Figure 7.6. Numbers of CD4 and CD8 cell per spleen in iNOS-deficient (-/-) and control (+/-) mice, either uninfected or on day 11 of infection with GUTat 7.2 trypanosomes.



Numbers of mononuclear splenocytes/spleen were determined by the trituration, centrifugation on 'Nycoprep' gradients and direct enumeration. Double labelling for CD4 and CD8 cells in the mononuclear splenocyte populations was then undertaken and proporations of each cell type determined by FACS analysis. The mean of 4 mice/group are shown ± 2 SE with 5000 cells counted by FACS for each sample.

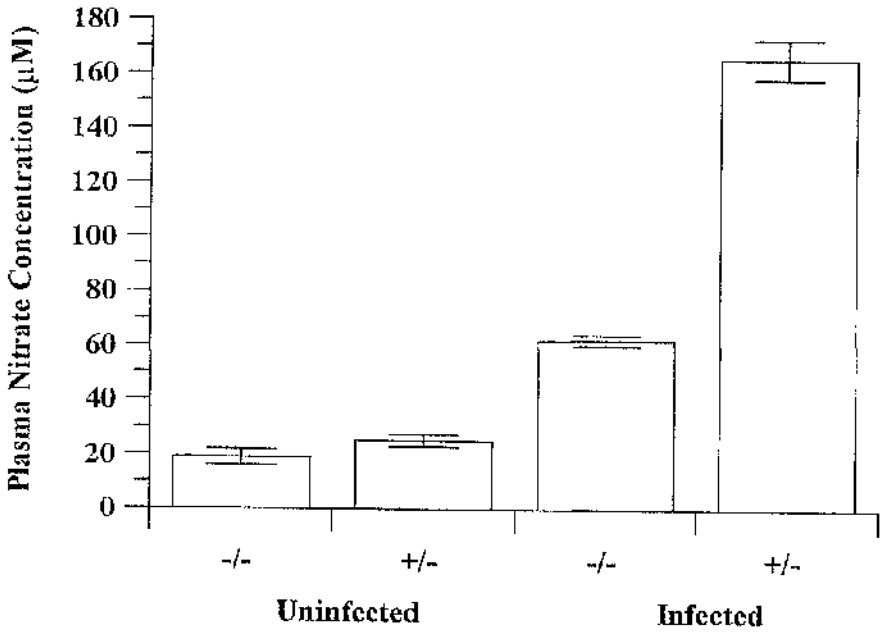
Figure 7.7. Splenic architecture of control and iNOS-deficient mice either uninfected or on day 11 of infection with GUTat 7.2 trypanosomes.





Sections of spleens from the control and iNOS-deficient mice were cut and stained with Haematoxylin and Eosin. The sections from control mice, uninfected (**a**) or chronically-infected (**b**) were examined at low maginfication (×10). The splenic architecture of the iNOS-deficient mice was also examined at low magnification in uninfected (**c**) or chronically-infected (**d**) mice. The architecture of the spleens uninfected (**e**) and from chronically-infected (**f**) mice were also compared at high magnification (×40). The sections were kindly cut and stained by Kate Orr, Division of Environmental and Evolutionary Biology, I.B.L.S., University of Glasgow.

Figure 7.8. Nitrate concentrations in plasma samples from control (+/-) and iNOS-deficient (-/-) mice either uninfected or on day 11 of infection with GUTat 7.2 trypanosomes.



Plasma nitrate levels were determined by the Greiss Reaction using the samples taken from individual mice. Results are expressed as the geometric mean ± 2 SE. This work was carried out by Dr. Jerry Stemberg, Department of Zoology, University of Aberdeen.

CHAPTER 8

GENERAL DISCUSSION

The main aims of my thesis were to investigate the possibility of a theoretical and experimental basis for antigenic variation as a mechanism for evading T-cell responses as well as a theoretical and experimental foundation for identifying T-cell epitopes common to several VSGs. This work involved devising a reliable *in vitro* assay system to examine T-cell proliferative responses against trypanosome VATs; investigating a number of different immunisation and infection regimes; predicting potential T-cell epitopes within VSG sequences and mapping these onto the tertiary VSG structure. As immunosuppression is the norm during trypanosome infections, I also investigated the regulation of T-cell responses with regards to NO during chronic infections.

There were two main difficulties in analysing T-cell responses during trypanosome infections. Firstly, Nude mice can control and survive infection suggesting that the antibody response is T-cell independent (Campbell *et al*, 1978) and secondly, the more recent literature characterising the type of T-cell response generated is confused: in one study VSG-specific T-helper 1 cells were identified, but only in the peritoneum and without proliferative ability (Schleifer *et al*,1993) whereas another group have identified $CD8^+$ cells as being crucial for IFNγ production and regulation of infection (Bakhiet *et al*,1993). My approach to addressing these difficulties was relatively straightforward. Harvesting the spleens at the first peak of parasitaemia allowed proliferative responses to be examined before the cells became unresponsive due to profound immunosuppression, and allowed examination of VAT-specific responses in infections.

The results in this thesis have identified a T-helper 1 type immune response during *T.brucei* infections. Not only was there IFNγ and IL-2 production but also mitogen-driven and trypanosome antigen-driven T-cell proliferation which has previously gone undetected. With the help of the "TSites" package, in predicting possible T-cell epitopes on the VSG sequences, and mapping of these selected epitopes onto tertiary structures, a number of these epitopes were found to be subsurface and highly variable. Because of

the tight packing of the VSG molecules on the surface of live trypanosomes, antibodies cannot come into contact with these regions within the individual VSG molecules (Blum *et al*,1993).

Following the first peak of parasitaemia there will be a large amount of shed VSG and other trypanosome antigens in the host (Diffley & Jayawardena,1982). It is possible that this large amount of antigen and the continual exposure to it will result in the mammalian host becoming anergic. I have obtained supporting evidence for the development of anergy in that it was possible to detect trypanosome antigen-driven proliferative responses at the first peak of parasitaemia (Chapter 4) but the proliferative response waned as infection continued and became more chronic (Chapter 7) as did the levels of IL-2 production (Chapter 4). It is reasonable to assume that if antigen-specific cells are present at the first peak of parasitaemia then they will be present as the infection continues. My data suggests therefore, that these cells have not been clonally deleted as the flow cytometry analyses in Chapter 7, examining chronic infections, showed the presence of these cell types in chronic-phase infections. The inability of these cells to produce IL-2 is also consistant with anergy as is the fact that the addition of exogenous IL-2 can restore lymphocyte responsiveness (Sileghem *et al*,1996). Clonal exhaustion rather than clonal deletion appears to be best explanation of the observed anergy during trypanosome infections. Soluble VSG is less immunogenic than membrane-bound VSG (Diffley,1985) and may be processed and presented to the T-cells in a different manner resulting in a lack of costimulation and therefore an insufficient response. Nothing is known about costimulation of responses to trypanosome infections. The way in which trypanosome antigens are presented after the first parasitaemic peak may determine the class of T-cells and cytokines, if any, that are produced, in turn leading to an ineffective response.

I have shown that NO has a major role in regulating the T-cell response during *T.brucei* infections in the murine model. This NO, from activated macrophages, influences the proliferative response as well as cytokine production in infected mice by reducing the responses compared with infected mice lacking iNOS. The mechanism as to how this NO acts is not yet known. NO has been shown to inactivate iron-dependent

enzymes in cells resulting in disruption of replication, respiration and energy production (Green & Nacy, 1993). Others have shown that NO causes a disruption in iron homeostasis which corresponds with decreased protein synthesis but not necessarily cell death (Liew & Cox, 1991). I would therefore suggest that during *T.brucei* infections, iron homeostasis is disrupted which in turn decreases protein synthesis and produces a reduced proliferative response to stimulation. This could be investigated in a number of small experiments. Groups of uninfected mice and mice in the chronic phase of parasitaemia could be used. Adding Desferrioxamine, in a range of concentrations, as an iron chelator that binds iron very tightly, to culture media would allow an *in vitro* comparison of proliferation, determined by ^{3}H-Thymidine incorporation, between mononuclear splenocytes from uninfected mice and chronically infected mice and a comparison with cultures which were not treated with Desferroxamine. Examination of the proliferation by the addition of exogenous iron in the form of transferrin or from iron nitrilotriacetate, an iron donor, should then be investigated to see if the addition of iron *in vitro* restores proliferation. If there is some restoration of the response then it would also be worthwhile saturating cultures of cells derived from chronically-infected mice with iron to see if it is possible to reverse some of the suppression by overloading with iron.

A connection between iron and NO could be further investigated by radiolabelling transferrin with ^{59}Fe and saturating cultures of cells from infected and uninfected mice for a few days. Following these incubations, fresh media, minus iron, would need to be added to the cells and the cultures incubated for several hours with different concentrations of SNAP or sodium nitroprusside as NO donors. Samples would then be removed at different time points and the amount of radiolabelled iron released into the culture supernatants from the cells and in the cell pellets could be determined. By using these data in conjunction with the proliferation data it will be possible to see if the lack of response is due to cytostasis. It may be that trypanosomes do not require as much iron as the host for cell functions and therefore targeting iron-dependent enzymes will be of benefit to the parasites.

Macrophages are the main source of TNFα and it is possible that TNFα acts as a physiological regulator of iron metabolism and it is only when large amounts are produced that abnormal iron retention occurs (Alvarez-Hernandez *et al*,1989). This could be the case during *T.brucei* infections whereby trypanosomes induce a large production of IFNγ and TNFα. These two cytokines then act on the macrophages which produced NO and together the NO and TNFα affect the levels of iron present.

As previously stated, antigenic variation is a mechanism to evade immune responses. One possibility which appears to have been little explored is that VATs or, more specifically, peptides within VATs, may differ in their immunogenicity. It may be that the sophisticated switching process is to prevent an immune response against one VAT which is more immunogenic than that against another. The switch might replace highly immunogenic peptides with peptides which induce a less effective response thereby escaping immune detection and continuing the infection. The trypanosomes may alter one of the key residues in a peptide which is essential for inducing an effective immune response. Altering the amino acid sequence in a VSG peptide could be adventageous to the pathogen as the MHC, processed peptide and T-cell receptor interaction may still occur but without the necessary costimulatory signals. The lymphocyte response can be inhibited during Hepatitis B infections, for instance, as the virus has the ability to alter epitopes which result in a lack of response to wild type viral epitopes (Bertoletti *et al*,1994). An extremely strong immune response against Hepatitis B antigens was observed in chronically infected patients but there was a failure to clear these infections which could be due to a number of consequences of the altered peptides, like the inhibition of IFNγ production which was not observed with the wild type peptides.

In the context of trypanosome infections, a great deal of knowledge would be gained by a series of immunisations of mice using overlapping peptides from the VSG sequences in combination with conjugate and adjuvant, then infecting groups of mice with different trypanosome VATs. This would identify conclusively which peptides were most immunogenic and which provide cross-protection against two or more VATs. Closer examination of the peptides which provided protection against infection could follow by substituting amino acids one by one to identify the essential residues required to provide

an effective immune response against the trypanosomes. This approach would be very time consuming and expensive but well worthwhile in the long term as regards a potential peptide vaccine against a number of VATs. A reasonable end goal of such a project would be to lower parasitaemia thus ameliorating the virulence of infection. Whilst such a result would be insufficient in humans it might be quite acceptable in cattle.

In conclusion, I have successfully developed an *in vitro* assay system which allowed me to examine T-cell proliferative responses during *T.brucei* infections. I was therefore able to examine trypanosome antigen-driven proliferation and cytokine responses in a number of infection and immunisation regimes, using paraformaldehyde-fixed trypanosomes or peptide conjugates for immunisation procedures, and thus define a role for T-cells during these infections. At the first peak of parasitaemia, a T_H1 response was generated and this was partially variant-specific. Following the first peak of parasitaemia, during the chronic phase of the disease when immunodepression occured, I determined a key role for NO in the regulation of T-cell responses during trypanosome infections with the NO appearing to have an important role with regards to parasite killing *in vivo* and in depressing the proliferative T-cell responses as well as IFNγ production *in vitro*. The multiple changes in immune function observed during trypanosomiasis cannot be assigned to a single event but reflect a number of host-parasite interactions and changes in host-derived mediators. It is not a single component of the host or of the parasite which is responsible for the continuation of infection, causing morbidity and mortality, but a combination of components. However, it is my opinion that the understanding of the mechanism by which NO operates and what it targets would be a substantial benefit to understanding immunosuppression during trypanosomiasis.

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