INHIBITION OF PARASITE GROWTH IN CHRONIC *TRYPANOSOMA BRUCEI* INFECTIONS

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Declaration

This thesis is submitted in accordance with the regulations for the degree of Master of Science in the University of Glasgow. Except where otherwise indicated, the results contained herein comprise solely my own work.

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Abbreviations

AnTat:	Antwerp *Trypanozoon* antigen type
B-ES:	bloodstream expression site
BSCM:	Bloodstream Culture Medium
CBSS:	Carter's Balanced Salt Solution
CNS:	central nervous system
DAPI:	4,6-Diamidino-2-phenylindole
DFMO:	difluoromethylornithine
DNA:	deoxyribonucleic acid
D-NAME:	N-nitro-D-arginine methyl ester
EATRO:	East African Trypanosomiasis Research Organisation
EGF:	epidermal growth factor
ELC:	expression linked copies
EMS:	ethylmethanesulphonate
FAD:	flavine adenine dinucleotide
FCS:	foetal calf serum
FGF:	fibroblast growth factor
FITC:	fluorescein isothiocyanate
GMP:	guanosine monophosphate
GUTat 7.2	Glasgow University *Trypanozoon* antigen type 7.2
HEPES:	N-[2-Hydroxyethyl]piperazine-N-[2-ethanesulphonic acid]
IFNγ:	interferon gamma

iNOS:	inducible nitric oxide synthase
kDa:	kilodaltons
L-NAME:	N-nitro-L-arginine methyl ester
L-NMMA:	N-monomethyl-L-arginine
log:	log to the base 10
MNC:	mononuclear cell
MW:	molecular weight
NADH:	nicotinamide adenine dinucleotide, reduced form
NADPH:	nicotinamide adenine dinucleotide phosphate, reduced form
NK cells:	Natural Killer cells
NMDA:	N-methyl-D-aspartate
NO:	nitric oxide
NOS:	nitric oxide synthase
NZ-FCS:	New Zealand foetal calf serum
OD:	absorbance
Oz-FCS:	Australian foetal calf serum
PBS:	phosphate buffered saline
PGE_2:	prostaglandin E_2
rIL-2:	recombinant interleukin 2
RNA:	ribonucleic acid
STIB:	Swiss Tropical Institute Basil
SNAP:	S-nitroso-N-acetyl-penicillamine

TLTF:	trypanosome-derived lymphocyte triggering factor
TM-1:	trypanosome medium-1
TNFα:	tumour necrosis factor alpha
WHO:	World Health Organisation
VAT:	Variable Antigen Type
VSG:	Variant Surface Glycoprotein

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SUMMARY

The objective of this project was to investigate the origin of inhibition of growth of trypanosomes in chronic infections as demonstrated by Turner *et al* (1996). In order to do so the following strategies were employed; adoptive transfer of splenocytes from chronically-infected to uninfected mice, inhibition of inducible nitric oxide (NO) synthase activity *in vivo* followed by infection with *Trypanosoma brucei* and infection of mice lacking inducible NO synthase activity.

The origin of growth inhibition was investigated using spleen cell transfer assays with a view to determining whether a putative inhibitory factor might be of parasite or of host origin. In irradiated recipients, spleen cells from trypanosome-infected donors caused inhibition of growth of subsequent infections but this observation was unreliable in repeat experiments. Inability to display a clear trend may have been due to resident macrophages of the recipient immune system being present and active in recipient mice following irradiation. Any effect upon growth of trypanosomes exerted by transferred macrophages *in vivo* might perhaps have been masked in recipient mice by resident macrophages.
In view of the dearth of literature regarding the actions of macrophage-produced NO upon the growth and survival of trypanosomes, the possible involvement of NO in the phenomenon of growth inhibition was investigated. The production of NO was shown to be increased upon infection with *Trypanosoma brucei*, but to an extent lower by several orders of magnitude than that demonstrated previously. This was concluded to be due to procedural inaccuracies in the work of others. Inhibition of inducible NO synthase activity was found to be without clear effect upon the growth rate of acute-phase trypanosome

infections or inhibition of growth of trypanosomes in chronic infection. A trend toward reduction in growth inhibition was demonstrated in mice which had received the iNOS inhibitor L-NAME but this observation was not repeatable. The use of mice genetically manipulated to lack inducible NO synthase activity also failed to demonstrate any effect of NO upon growth inhibition in chronic infections. A reason for my failure to demonstrate a role for NO in regulation of trypanosome growth could be that L-NAME treatment of mice and iNOS gene deletion were found to be ineffective in achieving a state of significantly reduced NO synthase activity in mice. Hence, whilst there was a failure to demonstrate a role for NO in growth inhibition, a lack of involvement for NO was not demonstrated either.

CHAPTER 1

GENERAL INTRODUCTION

1. General Introduction

1.1 Disease and Species

African Trypanosomiasis, a disease of both humans and animals, has maintained a strangle-hold on development in Africa throughout this century. In livestock, infection causes nagana, characterised by slow growth, weight loss, poor milk yield, infertility, abortion and impaired ability to work as draft and transport animals. In humans the disease is known as Sleeping Sickness and, if untreated, is generally fatal. African trypanosomes are also harboured by animals indigenous to Africa but with no apparent ill affects. Reservoir hosts of trypanosomes such as these support a constant population of the parasite which is readily transferred to domestic animals and humans (Vickerman 1993 *et al*).

The disease occurs in 36 African countries between the latitudes of 14° North and 29° South. Some 50 million people are at risk of infection with 25 000 new cases reported annually although this is thought to be an underestimate (Kuzoe 1993). The tsetse fly vector infests 11 million km^2 south of the Sahara (Vickerman 1993 *et al*). The history of African Trypanosomiasis is characterised by epidemics, periods of low endemicity followed by resurgence and outbreaks. The disease was brought partially under control in the 1950s in West and Central Africa, however following independence of most African countries sleeping sickness has returned due to failure by national health authorities to give the disease adequate priority, lack of adequate resources, political and civil unrest.

Recrudescence of old foci and geographical spread have been reported in recent years in Cameroon, Chad, Congo and the Republic of Central Africa (WHO 1990). A potential risk of a major epidemic in the near future is believed to exist in Zaire where 10 000 cases have been reported annually and external technical and financial aid has been withdrawn (Kuzoe 1993).

Control of African trypanosomiasis falls broadly into three main approaches; disease management through drugs and/or vaccination, use of trypanotolerant cattle and vector (tsetse fly) control. Trypanosomiasis in cattle and humans can be readily treated using drugs. In the case of cattle, whilst trypanocidal drugs are cost-effective, resistance to drugs in certain countries has become a significant problem (Barrett 1997). Drug treatment of human trypanosomiasis has met with moderate success and will be discussed in more detail later. Extensive efforts have been invested in the search for a vaccine for trypanosomiasis but little success has been experienced due to the number of different serodemes present and the occurrence of antigenic variation (Vickerman 1985).

Trypanotolerant breeds of cattle exist in certain parts of Africa and are suited to areas of low to medium prevalence. Productivity is lost, however, as the animals are subject to chronic infection and may succumb to the disease under high challenge. Control of tsetse flies has been employed in the past with considerable success. Spraying infested areas with pesticides is cost effective and has proved to be less objectionable than the previously employed strategy of shooting wild animals, which represented reservoirs of the parasite, in belts around livestock production areas. A major advance in the fight to control tsetse flies has been the application of bait technology whereby tsetse are lured to live or artificial

bait in order to kill or sterilise them. This technique has been shown to be cost competitive with other techniques (Barrett 1997) and effective in reducing epidemiological risk to humans living in endemic areas (Laveissiere *et al* 1994). Control of the vector, whilst an effective method of combating the disease, is reliant upon substantial resources from national health authorities, political and civil stability and in the case of trapping of tsetse, consistent contribution of labour by farmers in control areas.

Trypanosoma species that infect man belong to the following species; *Trypanosoma brucei*, *T.cruzi* and *T.rangeli*. The subspecies *T.b.rhodesiense* and *T.b.gambiense* are transmitted by *Glossina morsitans* and *G. palpalis* respectively, both commonly known as the tsetse fly, and cause sleeping sickness in East Africa and Western and Central parts of equatorial Africa respectively. *T.cruzi*, transmitted by the reduvid bugs of the genera *Triatoma, Panstrongylus* and *Rhodnius*, is the causative agent of Chagas' disease in Central and South America. *T.rangeli* also occurs in South and Central America (Zaman and Keong 1990).

T.congolense and *T.vivax* and the subspecies, *T.b.brucei*, are parasites of native antelopes and other African ruminants and cause nagana in non-indigenous livestock including pigs, sheep, goats, oxen, horses, camels, dogs, donkeys and mules. Humans are not susceptible. These species are all transmitted by *Glossina* flies. In areas outside of the tsetse belt *T.evansi* can be an important pathogen of camels, horses and cattle. It is mechanically transmitted by tabanid flies. *T.equiperdum* affects horses and is transmitted venereally (Vickerman *et al* 1993).

1.2 Life Cycle and Cell Cycle

Infection with *Trypanosoma brucei* occurs when an infected tsetse fly feeds upon the blood of an uninfected host and trypanosomes enter the host in the saliva of the fly. Only the metacyclic stage is able to initiate infection. Infection is characterised by undulating parasitaemias consisting of a phase of increasing parasite numbers due to multiplication of slender forms and remission due to immune clearance of parasites from the blood. *T.brucei* has two morphologically distinct forms in the blood; long slender forms capable of multiplication and shorter stumpy forms which do not undergo division. Both forms are covered by a variant surface glycoprotein (VSG) coat.

Long slender trypanosomes exhibit an elongated nucleus, a narrow and elongated posterior end, a kinetoplast distal from the posterior end and a long free flagellum (Wijers 1960). This form of trypanosome metabolises glucose in the blood of the host and excretes pyruvate. Mitochondrial enzyme activity is repressed and mitochondria lack cristae in this form of the parasite (Vickerman 1985).

Stumpy forms of trypanosomes have a nucleus which is either oval or round. The posterior end is blunt or pointed to one side, the kinetoplast either terminal or subterminal and the trypanosome considerably broader than slender forms. Cytoplasm is often visible between the nucleus and the plasma membrane. A free flagellum is nearly always absent (Wijers 1960). As slender forms transform into stumpies the mitochondria swell and develop tubular cristae concomitant with the acquisition of proline and α-ketoglutarate

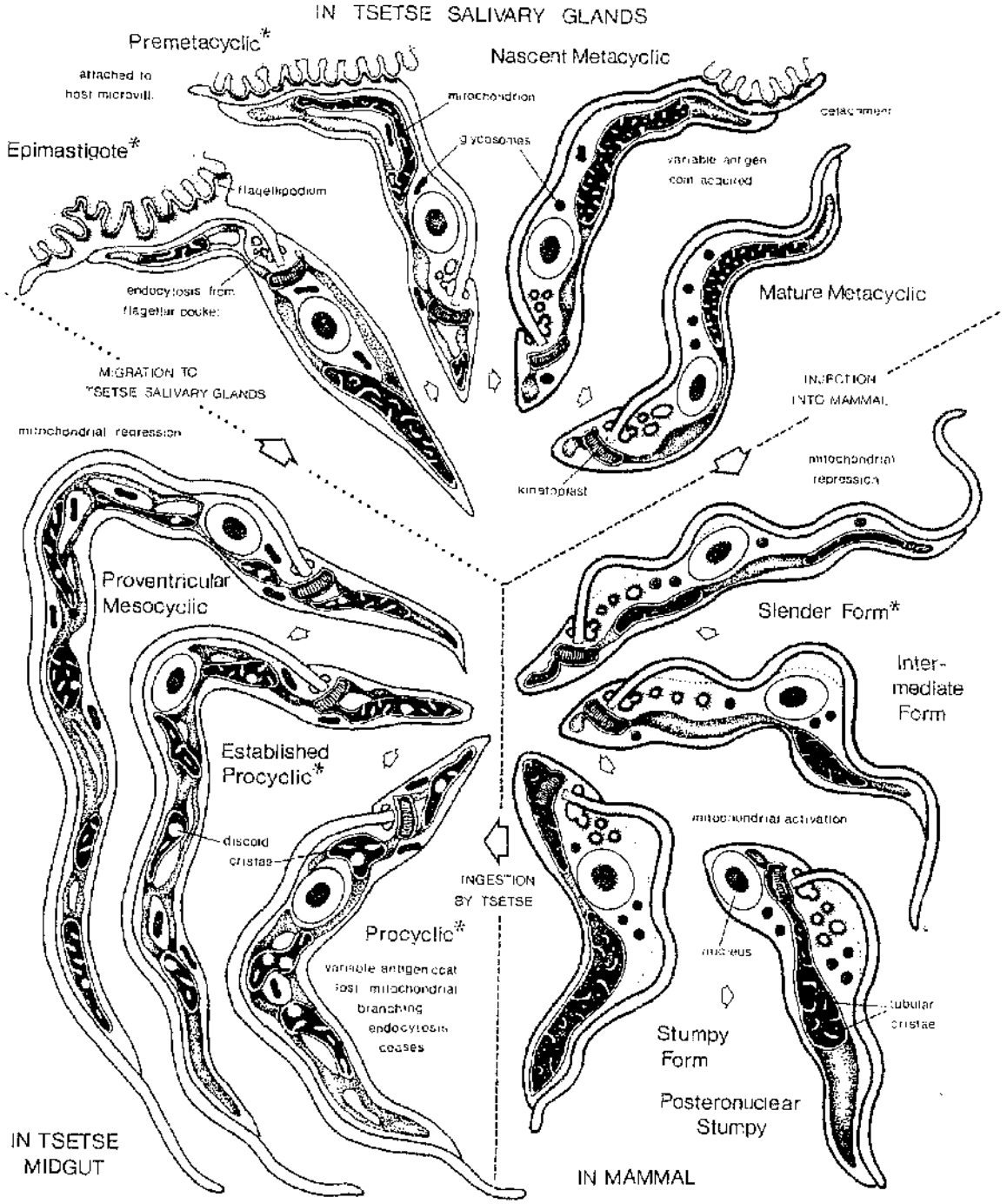


Diagram 1 Trypanosome life cycle.
Reproduced from; **Vickerman** (1985)Developmental Cycles and Biology of Pathogenic Trypanosomes. *British Medical Bulletin*. **41**:107. '*' multiplication occurs in these stages.

oxidase activity. These changes herald the switch to amino acid (mainly proline) based energy metabolism that occurs in the fly gut, diagram.1 (Vickerman 1985).

Slender forms give rise to an increasing parasitaemia composed predominantly of parasites with identical VSG surface coats. As parasite numbers increase in the blood, slender forms undergo morphological change into first intermediate forms with characteristics of both forms then into the short stumpy forms. Stumpy forms are more resistant to immune killing than slender forms and hence constitute the major proportion of the parasites when parasite numbers are decreasing. Slender forms, when ingested by a feeding tsetse fly, rapidly die in the fly gut due to lack of glucose. Stumpy forms survive however and undergo changes to become procyclic, Diagram 1. The VSG coat is lost upon transformation to procyclic forms (Barry and Vickerman 1979, Turner *et al* 1988). Procyclics divide by binary fission and establish an infection in the tsetse midgut. They give rise to proventricular forms that are responsible for migration to the salivary glands of the fly. Here, the non-dividing proventricular forms transform to multiplicative epimastigote forms which are attached to the salivary epithelium by the flagellum. The epimastigotes differentiate via the premetacyclic and nascent metacyclic forms, both of which are attached by the flagellum to the epithelium, into the non-dividing mature metacyclic trypomastigote. At this point trypomastigotes re-aquire VSG coats. Metacyclics are released from the epithelium to swim free in the saliva and are injected into the host in tsetse saliva upon feeding (Vickerman *et al* 1993, Vickerman 1985, Zaman and Keong 1990).

Trypanosomes replicate by binary fission. The cell cycle of trypanosomes is unusual in that, in common with other kinetoplastids, the trypanosome cell has three single-copy organelles; the nucleus, the kinetoplast and a flagellum anchored to the basal body, the replication of which must be co-ordinated. Each organelle must be replicated once and once only before segregation into daughter cells. Whilst diploid chromosomes in the nucleus segregate faithfully, as does the DNA in the kinetoplast, nuclear intermediate and minichromosomes do not (Tait and Turner 1990). Study of cell cycle events to date has been confined to procyclic forms of *T. brucei* maintained *in vitro*. Several features of the trypanosome cell cycle have been established. Whilst replication of the basal body and initiation of the DNA synthesis phase (S phase) for the nucleus and kinetoplast all coincide, timing of termination of this phase differs between the nucleus and kinetoplast. Timing of nuclear mitosis and kinetoplast division also differs. It has been shown that there is an extended phase of post mitotic cytokinesis (Sherwin and Gull 1989, Woodward and Gull 1990). Kinetoplast division has been shown to occur at the same time, or after mitosis in different species of trypanosomatid (Simpson 1972). After replication has been achieved, organelles undergo migration to span a division furrow initiated at the anterior end of the cell which then splits longitudinally into two daughter cells (Sherwin and Gull 1989). Migration of organelles is mediated by microtubules (Matthews *et al* 1995, Robinson *et al* 1995). Metacyclic and stumpy cells do not undergo replication and are believed to be arrested in G_0 of the cell cycle (Shapiro *et al* 1984).

1.3 Course of Infection, Pathology and Drug Treatment

Infection of the mammalian host occurs when the bite of an infected fly deposits metacyclic trypanosomes in dermal connective tissue. After about three days a local inflammatory reaction occurs in the skin - the chancre. The chancre development peaks by day six post infection and then subsides. From the chancre trypanosomes enter the draining lymphatic and then the bloodstream. Trypanosomes appear in the lymph prior to macroscopic development of the chancre (Barry and Emery 1984). Metacyclic trypanosomes undergo morphological change to emerge as elongate replicative forms characteristic of the bloodstream stage of the life cycle. *T.brucei* may secondarily escape the bloodstream and enter the tissues to multiply in the tissue fluid. In chronic infections of *T.brucei* trypanosomes invade the brain and cerebrospinal fluid (Vickerman 1993 *et al*) and parasites in this location have been implicated as a source of relapsing infection following chemotherapy (Jennings *et al* 1979, Poltera 1985).

The literature documenting the pathology of African trypanosomiasis, caused by *T.brucei*, is extensive and is complicated by the nature of many of the publications in dealing with only one aspect of the disease. Pathology will here be described in a sequential manner following infection. Upon infection by infected fly bite a chancre develops at the bite site. Chancres are characterised by marked oedema and intense cellular infiltration with proliferating endothelial cells, fibroblasts and trypanosomes (Poltera 1985). Trypanosomes may leave the chancre to enter the systemic circulation either by direct migration by dermal venules or via the lymphatic system and the thoracic duct (Ssenyonga

and Adam 1975). Changes in the lymph nodes include follicular hyperplasia, sinus histiocytosis, perivascular mononuclear infiltration, reduction in lymph cell population and partial obstruction of lymph flow (Poltera 1980). Anaemia is often observed in humans during infection and is thought to be due to formation of immune complexes followed by complement mediated haemolysis (Woodruff *et al* 1973). More recently anaemia has been postulated to be due to impaired erythropoeisis in the bone marrow (Mabbot and Sternberg 1995). Non-specific proliferation of B cells, and subsequent hypergammaglobulinaemia, is a dominant pathological event in the blood (Greenwood and Whittle 1980, Lambert *et al* 1981) and will be discussed in more detail in the context of immune dysfunction in a later section. Platelets have been shown to have a reduced life span (Robins-Browne *et al* 1975) and thrombocytopenia results. Immune complexes lead to oedema, inflammation and hypotension. Hypocomplementaemia and low C3 levels indicate activation of the complement system (Greenwood and Whittle 1980, Lambert *et al* 1981). Renal damage is thought to involve T cells and macrophages (Van Velthuysen *et al* 1994). Pancarditis has been described in man (Poltera *et al* 1976) and underlies clinically reported heart failure in sleeping sickness patients. Other pathological effects of the disease include diarrhoea, hypoglycaemia, hepatosplenomegally, impotence, amenorrhoea, extensive endocrine dysfunction (Reincke *et al* 1993) and, in late stage trypanosomiasis, CNS dysfunction. In late stage trypanosomiasis, trypanosomes invade the CNS. Damage occurs to the blood-brain barrier with invasion of B cells, antibody formation and immune complexes in the cerebrospinal fluid (Greewood and Whittle 1980, Lambert *et al* 1981) Clinically, symptoms range from headache to psychiatric problems and coma. Meningoencephalitis in

trypanosomiasis is characterised by severe vasculitis and, at a cellular level, destruction of neurones, microglial reaction, diffuse scattering of inflammatory cells in the neuropil and foci of demyelination (Poltera 1985).

There are currently four drugs available for the treatment of African trypanosomiasis in humans; eflornithine, suramin, pentamidine and melarsoprol. The last three drugs have all been in use for more than 40 years. Drug treatment of trypanosomiasis is complicated due to poor drug absorption, toxic side effects and the emergence of drug resistant strains of the parasite.

Suramin, the drug of choice for early-stage trypanosomiasis, especially *T.b. rhodesiense* infections, is ineffective in late stages of infection due to its inability to cross the blood brain barrier, or indeed any lipid bilayer. Suramin is thought to enter the trypanosome bound to serum proteins which are taken up by receptor mediated endocytosis (Wang 1995). The mechanism by which suramin exerts its antitrypanosomal activity is as yet unknown. Although suramin is a potent inhibitor of all the glycolytic enzymes in *T. brucei* (Wilson *et al* 1993), it is unlikely that this action is responsible for suramin's trypanocidal activity as these enzymes are all confined to glycosomes and hence inaccessible to suramin. The genes encoding these enzymes are located in the nucleus, hence the enzymes are synthesised and transported to the glycosome without any proteolytic modification (Hart *et al* 1987). It has been suggested that suramin may bind to these enzymes in the cytoplasm and interfere with their import to the glycosome, hence causing a time dependent

reduction in their concentration (Wang 1995). In 70 years of treating trypanosomiasis trypanosomal resistance to suramin has not posed a serious problem.

Pentamidine, introduced in 1937, is effective against early stage *T. brucei gambiense* but drug resistance has arisen in some strains of trypanosome (Kuzoe 1993). Its mechanism of antitrypanosomal action is unknown. Possibilities include binding to nucleic acids (reviewed by Wang 1995) and disruption of kinetoplast DNA (reviewed by Newton 1974).

Melarsoprol was until 1990 the only drug capable of treating late stage *T. b. gambiense* and *T. b. rhodesiense* infections. It is argued to exert its antitrypanosomal effect by inhibiting several glycolytic enzymes (Wang 1995). Melarsoprol has two major problems associated with its use. Potentially fatal reactive encephalopathy occurs in 5 - 10% of patients treated and resistance has developed to melarsoprol in both *T. b. gambiense* and *T. b. rhodesiense* (Wang 1995).

Eflornithine, a relatively new drug introduced in 1990, is effective against early and late stage *T. brucei gambiense* infections. It is a potent suicide inhibitor of ornithine decarboxylase, the key enzyme in the pathway leading to biosynthesis of polyamines. Eflornithine (DFMO) is a relatively safe drug but has a low efficacy and short duration of action. It is also ineffective against *T.brucei rhodesiense* infections (Wang 1995). Whilst there have been no clinical reports to date of trypanosome resistance to DFMO, some isolates of *T.b.rhodesiense* which had never been exposed to DFMO were found to exhibit innate resistance to the drug (Bales *et al* 1989).

1.4. Trypanosome Immunology

The cellular immune response to infection appears rapidly in the form of the chancre which is caused not only by proliferating trypanosomes but also by an inflammatory immune response. Lymphocyte output from the chancre has been shown to increase 6 - 8 fold over normal levels by day 10 of infection with the number of blastocysts greatly increased (Barry and Emery 1984). Systemic immune responses to trypanosomes occur later and are responsible for the clearance of trypanosomes from the blood.

Trypanosomes are killed by antibody mediated lysis. The main antibody subclass responsible is IgM which is produced by activated B cells, (Seed 1977, Crowe *et al* 1984). Specific IgM antibody is produced rapidly in response to the appearance of trypanosomes in the blood. Specific IgM agglutinates trypanosomes and activates the alternative pathway of complement resulting in lysis of the trypanosomes (Flemmings and Diggs 1978, Ferrante and Allison 1983). Agglutinating activity has also been demonstrated in the IgG fraction of the serum of infected rabbits. This activity occurs later in infection and at a lower level than does agglutinating activity in the IgM fraction (Seed *et al* 1969). IgM has been shown to have greater agglutinating and, in the presence of complement, greater neutralising activity than IgG (Seed 1977).

Trypanosomes are also cleared from the circulation by antibody mediated cellular cytotoxicity. Trypanosome-specific antibodies bind to and opsonise the parasites leading to phagocytosis by Kupffer cells in the liver and by splenic macrophages (MacAskill *et al* 1980, 1981). Indeed the resistance of mice to infection with trypanosomes has been

shown to be increased non-specifically by treatment with immunostimulants such as *Propionibacterium acnes* (Black *et al* 1989) and the uptake of trypanosomes to be increased by treatment with either *P.acnes* (= *Corynebacterium parvum*) or *Mycobacterium bovis* (MacAskil *et al* 1980), which activate the mononuclear phagocyte system. An illustration of the significance of this mechanism for clearance of parasites is provided by work by Rurangirwa *et al* (1986) showing the phagocytic system of the trypanosome resistant Wildebeest (*Connochaetes taurinus*) to be superior to that of trypanosome susceptible cattle.

The multiphasic nature of trypanosome parasitaemias (Barry and Turner 1991) is due to two characteristics of the trypanosome. Firstly slender forms of trypanosomes have been demonstrated to be more susceptible to killing by antibody mediated complement dependent lysis than stumpy forms (McLintock *et al* 1993). Secondly, more importantly, trypanosomes undergo antigenic variation.

Each trypanosome cell is completely enwrapped in a surface coat that provides a physical barrier protecting the plasma membrane from attack by large molecules of the immune response such as immunoglobin and C3b. This surface coat consists of a monomolecular layer of a single species of glycoprotein, the VSG which is expressed at high copy number, greater than 10^7 molecules per cell. There are more than 1000 different VSGs that can be expressed and these are all antigenically distinct; i.e. each VSG is a different variable antigen type (VAT).

During the course of an infection, VATs are expressed in a 'semi-predictable' ordered sequence. As a sub-population of trypanosomes expressing a particular VAT gives rise to

patency and predominates an infection, VAT-specific immune responses are generated but in the time interval between immune recognition and killing, antigenic variation towards expression of new VATs, as yet unrecognised by the immune system, will have occurred causing prolongation of infection (Vickerman *et al* 1993). In tsetse transmitted infections, several VATs can be detected at any particular time point in an infection, though not with identical prevalence (Seed and Effron 1973, Turner *et al* 1986). After syringe-passaging and recloning however, trypanosome populations homogeneously express a single VAT. Although this laboratory artifact is biologically unrealistic in terms of the antigenic challenge it presents to the host, it is a very useful experimental simplification that has considerably aided our understanding of antigenic variation (Turner 1990).

Antigenic variation is thought to occur in a hierarchical fashion with some VSGs appearing earlier in infection than others. This hierarchy is believed to occur in a divergent manner whereby one VAT can switch to any of a few different VSGs (Barry and Turner 1991). The VSGs are encoded by over 1000 genes.

The differential activation of the different genes is achieved by five mechanisms. Formation of Expression Linked Copies (ELC) by gene conversion, in which the gene to be activated is copied into a Bloodstream Expression Site (B-ES) and replaces the ELC already present in the B-ES, is thought to be the most important mechanism for antigenic variation. Telomeric conversion is a process whereby telomeric genes are duplicated from non-homologous chromosomes into a B-ES and may be the main route for activation of minichromosomal genes. The third mechanism, which is the switching off of one B-ES at the promoter and switching on of another, is thought to be capable of activating only a

limited number of VSG genes but may predominate in the very early stages of infection. Two additional mechanisms include reciprocal recombination of VSG genes from one chromosome to another and, rarely, assembly of mosaic ELCs containing pieces of defective genes whose encoded epitopes have presumably not been experienced by the host. This last mechanism is only seen in advanced infections when there is considerable selective pressure for new VSG variants (Barry 1997).

The involvement of T cells in the immune response to trypanosomes infections has been suggested by several studies upon the course of infection in the absence of different subsets of T cells. Reduced parasitaemia and increased survival in congenitally athymic mice over heterozygous litter mates would suggest that resistance to *T.rhodesiense* infection is relatively independent of T cell function (Campbell *et al* 1978). In contrast, reconstitution studies of chimeric mice (De Gee and Mansfield 1984), the absence of a correlation between ability to control infection and antibody titre (De Gee *et al* 1988) and direct cloning of VAT-specific T cell lines from trypanosome-infected mice (Schleiffer *et al* 1993) all suggest that resistance to infection is at least partly T cell dependent. Human T cells have been shown to proliferate in response to exposure to *T.b.rhodesiense*. These cells display enhanced helper cell activity, but no suppresser activities, during mitogen induced antibody synthesis (Selkirk *et al* 1983). This would suggest a possible role for T cells in induction of the hypergammaglobulinaemia characteristic of infection with African trypanosomes.

More recently T cells have been found to have a pro-trypanosomal activity as indicated by their interactions with *T. b. brucei* in mice. Increased resistance to *T. b. brucei* in mice in which CD8 and CD4 molecules had been deleted (Rottenberg *et al* 1993) and reduced growth of *T. b. brucei* in rats with $CD8^+$ cells depleted (Bakhiet *et al* 1990) would suggest that $CD8^+$ and to a lesser degree $CD4^+$ cell activities may promote parasite growth. Rat mononuclear cells (MNC) have been shown to release IFNγ in a $CD8^+$ T cell dependent manner in response to *T. b. brucei* (Bakhiet *et al* 1990, Olsson *et al* 1991). Depletion of $CD8^+$ cells *in vitro* results in reduction of IFNγ production and a reduction in parasite growth (Bakhiet *et al* 1990). However $CD8^+$ T cells are not thought to have any effect upon control of trypanosomiasis in natural hosts (Sileghem and Naessens 1995). The effects of INFγ upon trypanosome growth will be discussed in depth in a later section.

Macrophages are capable of phagocytosing trypanosomes in the presence of VAT specific antibodies (McAskill *et al* 1981). They have also been implicated in the suppression of cellular immune responses described for this disease. Their role in immunosuppression will be discussed later.

Comparisons of the mean survival times of *T. b. gambiense* infected mice congenitally deficient for Natural Killer (NK) cells suggest that the presence of NK cells may be detrimental to the host although this has not been investigated in any depth (Jones and Hancock 1983).. In the same study mice deficient in macrophages displayed higher mortality than those with an intact macrophage system suggesting that, whilst lack of

macrophages does not significantly alter parasitaemia, an intact macrophage system is crucial to survival of *T.b.gambiense* infection (Jones and Hancock 1983).

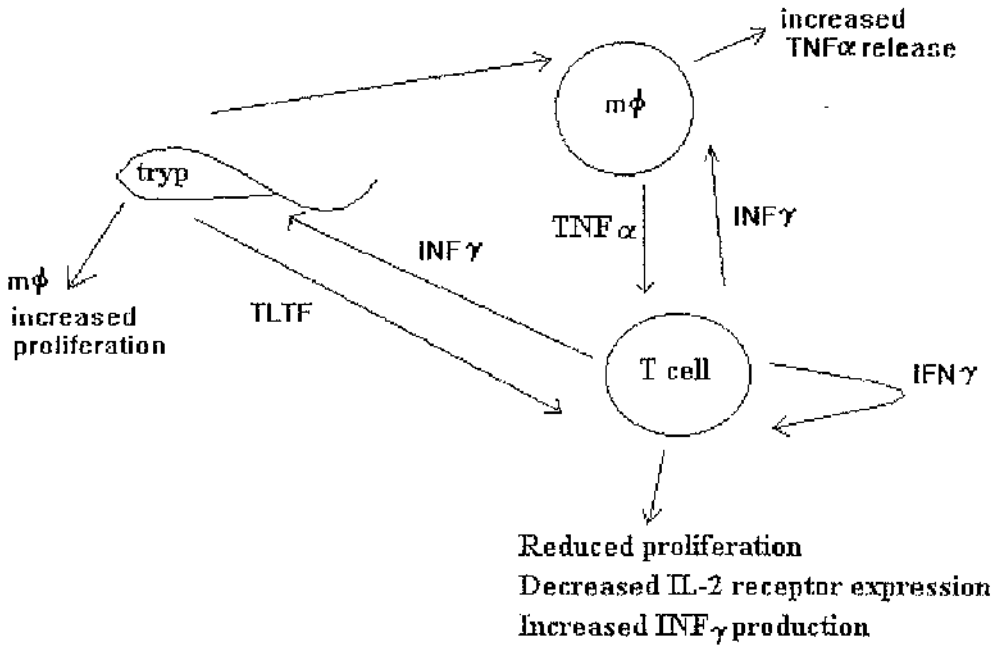
African trypanosomes evade the immune system by two separate and very effective strategies. The first of these is Antigenic Variation, a feature shared with malaria parasites (Brannan *et al* 1994). This mechanism has been discussed previously.

The second mechanism by which African trypanosomes evade the immune response is by inducing a generalised immunosuppression. Humoral and cellular immune responses are depressed to both trypanosomes and to organisms of secondary infections. It is often secondary infection that leads to death of the host (Askonas and Bancroft 1984). Upon infection a short-lived enhancement of antibody formation precedes immune dysfunction. Although hypergammaglobulinaemia is characteristic of this disease and levels of IgM become raised, much of this antibody appears to be non-specific. Formation of specific antibody declines with production of specific IgG declining more rapidly than production of IgM (Hudson *et al* 1976, Sacks and Askonas 1980). Hence in chronic infections the host responds with low levels of specific IgM antibody. B lymphocytes isolated from infected mice display an abherant phenotype including decreased levels of surface IgM and CD23 and increased levels of CD69 and l-selectin. The activation response of B cells early in infection precedes arrest in the cell cycle at G_0/G_A (Sacco *et al* 1994).
The degree of immunosuppression resulting from infection varies with the strain of trypanosome, stimulating speculation that immunosuppression may be a function of

parasite virulence (Nantulya *et al* 1982, Sacks *et al* 1980). An alternative explanation is that certain strains of trypanosomes are inherently more immunogenic than others, (Nantulya *et al* 1982), leading to immunosuppression by competition for immune response: antigenic competition. Injection of trypanosome membrane fractions from strains of differing virulence was found to result in greater depression of IgM antibody formation in the case of highly virulent strains. Depression of IgG production was not found to be affected by the virulence of the infecting strain. These findings are highly suggestive of a role for parasite virulence in determining degree of immunosuppression. However increasing the dose of membrane fraction was also found to increase the level of immunosuppression suggesting that antigenic competition is also occurring (Sacks *et al* 1980). The involvement of antigenic competition has been further indicated by investigating the level of immune response to separate strains of trypanosomes in mixed infections in mice. Mice were infected with two strains of differing virulence. The immune response was found to be depressed to a greater extent in response to the less virulent strain. The virulent strain which proliferated more induced a greater immune response suggesting that the level of immune response experienced is related to the amount of circulating antibody. These findings are suggestive of immune depression being a function of antigenic competition (Nantulya *et al* 1982).

T cells exhibit an altered phenotype during infection. Suppresser T cells have been described since the late 1970s (Jayawardena *et al* 1978), however it is only recently that the mechanisms underlying the generation of these cell has been elucidated. Lymph node cells from mice infected with *T.brucei* have been shown to contain macrophage-like cells

which inhibit T cell IL-2 receptor expression (Silegham *et al* 1989). T cells cultured in the presence of *T.b.rhodesiense* exhibit reduced proliferation and reduced IL-2 receptor expression. *T.b.rhodesiense* releases a soluble factor responsible for both prevention of T cells passing through the cell cycle and their altered phenotype (Sztein and Kierszenbaum 1991). Although T cells become non-proliferative during infection $CD8^+$ T cells also become hypersensitised to IFNγ production. This occurs concurrently with reduced proliferation and expression of the IL-2 receptor (Darji *et al* 1993; 1996). Addition of neutralising anti IFNγ antibodies reduces suppression of proliferative response and restores the level of IL-2 receptor expression. TNFα produced by macrophages is implicated in the hyperproduction of IFNγ by $CD8^+$ T cells (Darji *et al* 1996). This leads to the hypothesis that T cell released IFNγ generates macrophages of a suppresser phenotype which exert a suppressive effect upon T cells, diagram 2.



mϕ denotes macrophage, tryp denotes trypanosome

Diagram 2: Postulated mechanisms underlying the generation of T cells and macrophages of a suppresser phenotype.

Macrophages undergo many changes in phenotype in infected animals. After first peak of infection macrophages have a reduced surface expression of mannose, Fc and complement receptors. Release of H_2O_2 and plasminogen activating factor is increased. Surface Ia expression is increased by two thirds in *T.b.brucei* infected mice (reviewed by Askonas 1985) but not in *T.rhodesiense* infected mice (Bagasra *et al* 1981). Macrophages from normal mice release more prostacyclin than prostaglandin E_2 (PGE_2), but in macrophages from infected mice this ratio of prostaglandin production is reversed (Askonas 1985). Early in infection with *T.b.brucei* IL-1 and PGE_2 production is increased. Later in

infection no significant levels of prostaglandin production are detected (Askonas and Bancroft 1984).

Macrophages from infected mice reduce proliferation of T cells from uninfected mice both *in vivo* and *in vitro* (Borowy *et al* 1990). Macrophages transferred from mice exposed to irradiated *T. b. brucei* trypanosomes have been found to suppress T cell dependent antibody responses in normal mice *in vivo* suggesting an interference with T cell help to B cells (Grosskinsky and Askonas 1981). The mechanism by which these macrophages exert their suppressive effects upon T cells has received great interest in recent years. Sileghem *et al* (1989) discovered that macrophages induced reduction in IL-2 receptor expression caused by the release of prostaglandins. The involvement of nitric oxide in the suppressive activities of macrophages has also been indicated by several workers in the field. Addition of the L-arginine substrate analogue, L-NMMA, to cultures of macrophages reduced suppression of mitogen-induced proliferation of splenic leukocytes as did addition of red blood cells. Both of these interventions reduced the level of NO in culture. Nitrite production, as an indicator of NO production, correlated with suppresser macrophages activity (Mills 1991). NO involvement in suppression of T cell responses has been further illustrated by inhibition studies (Sternberg and McGuigan 1992, Schleifer and Mansfield 1993, Mabbott *et al* 1995, Sternberg and Mabbott 1996). TNFα and IFNγ are believed to be required for induction of NO production by activated macrophages (Schleifer and Mansfield 1993, Sternberg and Mabbott 1996).

1.5 Nitric Oxide

Nitric oxide (NO) is a small diffusable molecule produced by a wide variety of cells in the body. First described as Endothelial Derived Relaxing Factor, NO has received intense interest in recent years.

NO is synthesised from a terminal guanidino amino acid of L-arginine (Hibbs *et al* 1990) by the enzyme Nitric Oxide Synthase (NOS). Three isoforms of NOS are currently known. One is constitutive, cytosolic, Ca^{2+} /calmodulin dependent and once activated, by acetylcholine receptor activation, releases NO for only short periods of time. NO produced by this enzyme is responsible for a variety of functions including maintaining vascular homeostasis. The second isoform, activated by NMDA receptor activation, is Ca^{2+}/calmodulin dependent and is located on neurones. This isoform is responsible for neurotransmission (Lowenstein and Snyder 1992). One other isoform of NOS is induced in a wide variety of cells in response to cytokines and endotoxins. This induced isoform (iNOS) is cytosolic, Ca^{2+} independent and once activated, cells synthesise NO for long periods of time. NO produced in this way is a cytotoxic molecule for invading microorganisisms and tumour cells (Moncada *et al* 1991). Inducible NO synthase activity has been demonstrated, during endotoxaemia, in cells in almost every major organ in the body with the exception of the brain. Immune cells with iNOS activity include macrophages, neutrophils and granulocytes (Sato *et al* 1995). Widespread activation of

iNOS is thought to be responsible for the massive reduction of blood pressure and cardiovascular collapse of septic shock (Sato *et al* 1995, Marczin *et al* 1996).

NO is highly diffusable. NO released from a single point source for a period of 1 - 10 seconds has a sphere of influence of 200μm (Wood and Garthwaite 1994). In the context of a blood parasite such as *T.brucei*, the most important endogenous inhibitor of NO activity is oxyhaemoglobin which is a well documented scavenger of free NO (Goretski and Hollocher 1988, Lancaster 1994, Mabbott *et al* 1994, Jia *et al* 1996). As NO has a half life of 0.5 - 5 seconds, inactivation of NO has relatively minor effects on NOs actions as the rate of diffusion is fast (Wood and Garthwaite 1994). Hence although NO is classically considered to act in a paracrine fashion *in vivo* it diffuses to significant concentrations at distances relatively far removed from a single point source of NO. Localised areas of vascularisation are capable of scavenging NO at distances many cell diameters away from the point of release of NO (Lancaster 1994). This causes a paradox in that if haemoglobin scavenges nitric oxide, how does NO exert it's actions at a distance from it's point of synthesis? One answer may come from recent work that has demonstrated the presence of nitrosothiols in the blood which are not scavenged by oxyhaemoglobin and have the same vasodilatory properties as free NO. Nitrosothiols could therefore be expected to deliver similar NO-like cytotoxic properties in the presence of oxy-haemoglobin (Jia *et al* 1996).

Nitric oxide is released from immune cells in response to cytokine activation and contact with endotoxins. It is one of the main cytotoxic molecules released by macrophages,

neutrophils and granulocytes in response to INFγ, TNFα and endotoxins and is part of the host defence against invading micro-organisms and tumour cells (Liew and Cox 1991, Abbas *et al* 1994). NO also has cytostatic activity. It is not yet clear how NO exerts it's cytotoxic and cytostatic activities but one possibility is that it acts by reacting with iron groups in enzymes in target cells (Drapier *et al* 1991, James 1995). This action is characterised by an iron loss from enzymes such as NADH dehyrogenase and succinate dehydrogenase (Wharton *et al* 1988) and degradation of enzymes of the electron transport chain (Liew and Cox 1991). In addition, NO can react with superoxide anions to form peroxynitrite anions which rapidly decay to release the highly reactive hydroxyl radicals (James 1995).

NO has antiparasitic activities recorded in a wide variety of organisms. Macrophages kill *Schistosoma mansoni* larvae in a NO-dependent mechanism (Liew and Cox 1991). *Leishmania major* are obligate intracellular parasites of macrophages. NO has been implicated in cellular killing of leishmania parasites as treatment of infected mice with NO substrate inhibitors increases parasite load and causes an increase in size of cutaneous lesions (Liew *et al* 1990). Other parasites in which a cytotoxic action of NO has been demonstrated include *Toxoplasma gondii* (Liew and Cox 1991), *Trypanosoma musculi* (Albright *et al* 1994) and the intracellular forms of *Trypanosoma cruzi*. Cell types other than macrophages also release NO in parasitic infections and have antiparasitic actions. Activated endothelial cells are capable of killing schistosomes *in vitro* and hepatocytes exhibit NO-dependent cytotoxic activity towards *Plasmodium berghei* parasites *in vitro*

(James 1995). NO has recently been shown to increase resistance of mice to infection with *Brugia malayi*, one of the causative agents of human lymphatic filariasis although no cell type responsible was identified (Rajan *et al* 1996).

Macrophage-produced NO has a cytotoxic activity upon *Trypanosoma brucei*. Trypanosomes have been shown to fail to proliferate *in vitro* when treated with nitric oxide gas or exposed to activated macrophages. This effect was reversed when excess iron was provided (Vincendeau and Daulouede 1991, Vincendeau *et al* 1992). These data are consistent with the reported reactivity of NO with Fe-S groups in target enzymes. The cytostatic effect of activated macrophages has been clearly demonstrated to be due to release of nitric oxide as established by inhibition studies (Vincendeau and Daulouede 1991). The effect exerted upon bloodstream trypanosomes by nitric oxide has also received recent attention. Inhibition of inducible nitric oxide production *in vivo* by treatment of mice with the substrate analogue N-nitro-L-arginine methyl ester (L-NAME) resulted in a 50% reduction in peak parasitaemia. Paradoxically, addition of the nitric oxide donor, S-nitroso-N-acetyl-penicillamine (SNAP), to axenic cultures of trypanosomes inhibited proliferation by 75%. This was also evident in the presence of activated macrophages. Interestingly, addition of whole blood, at dilutions of up to 1 in 200, to these cultures totally abrogated the inhibition of proliferation caused by activated macrophages (Sternberg *et al* 1994). Oxyhaemoglobin is a scavenger of nitric oxide (Jia *et al* 1996, Lancaster 1994, Wood and Garthwaite 1994) and has been postulated to act as a

sink for macrophage-produced nitric oxide hence abrogating any anti-trypanosomal effects of nitric oxide *in vivo*.

1.6 Growth Regulation

The growth of parasites in a host is influenced by many factors. Amongst these are the nutritional status of the host, rate of multiplication of the parasite and the immune response of the host to the parasite. Cytokines, as a product of cellular immunity, have been shown to have numerous effects upon the life stages of various protozoan parasites. Interleukin-2 and Granulocyte/Macrophage Colony Stimulating Factor are growth factors for *Leishmania mexicana amazonensis* promastigote forms (Mazingue *et al* 1989, Charlab *et al* 1990). Indeed T cells, one of the main producers of IL-2 in the mammalian host, are known to have a growth promoting effect in leishmania infections. The growth promoting effect of IL-2 also occurs when promastigotes are introduced into mouse hosts, the size of lesions and the number of parasites isolated from the lesions are increased (Mazingue *et al* 1989). Fibroblast Growth Factor (FGF)-like proteins, reactive with anti-bovine-FGF monoclonal antibodies, have been demonstrated in procyclic culture forms of *Trypanosoma brucei rhodesiense* and *Leishmania donovani* promastigotes and may have a role in growth and host-parasite interactions (Kardami *et al* 1992). In contrast, interferon gamma (IFNγ) has been shown to be cytostatic to *Leishmania donovani* promastigotes (Bhattacharya *et al* 1993). Ovine recombinant IFNγ reduces replication of *Toxoplasma*

gondii inside ovine fibroblasts and, at a lower dose, alveolar macrophages (Oura *et al* 1993). Inhibition of intracellular replication of *T. gondii* has also been observed with macrophage-released TNFα acting in synergy with IFNγ (Langermans *et al* 1992). Recombinant IL-2 (rIL-2), IFNγ and TNFβ have also been shown to have growth inhibitory effects upon *Plasmodium falciparum* erythrocytic stages *in vitro*. Growth inhibition is a consequence of defective schizont maturation and is greatest in the case of rIL-2, followed by IFNγ and is least pronounced in the case of TNFβ. These cytokines are thought to be partly responsible for induction of degenerate intra-erythrocytic parasites (Orago and Facer 1993).

The direct effects of cytokines upon growth of infectious agents is not confined to eukaryotes. Interleukin-1, (IL-1), has been shown to have a growth promoting effect upon virulent strains of *Escherichia coli* and to bind to the *E. coli* cell in a manner suggestive of the existence of a specific receptor for IL-1. This binding does not occur with avirulent strains of *E. coli* and may underlie the basis of virulence of *E. coli* in the human host (Porat *et al* 1991). A growth promoting activity for IL-6 exists in intracellular stages of *Mycobacterium avium*. IL-6 increases bacterial growth and is thought to bind to a specific receptor on the surface of the cell (Denis 1992). The importance of findings of cytokine-determined regulation of parasite growth is considerable in the context of an infection. They are suggestive of parasite growth regulated by molecules that are active components of the host-parasite interaction; not simply limited by availability from the host of key nutrients.

T.brucei parasites will undoubtedly encounter a plethora of host cell derived factors in the bloodstream and tissues of the mammalian host. These factors may have effects upon trypanosomes ranging from promotion of growth to cytostasis and cytotoxicity. Bloodstream trypanosomes are unable to grow in culture in the absence of transferrin. Transferrin is thought to provide the iron required by the parasite for growth. A transferrin receptor-like protein has been demonstrated on the membranes of bloodstream form trypanosomes but not on the insect-borne stages. Transferrin is thought to be bound by the binding protein and internalised by endocytosis at the flagellar pocket (Coppens *et al* 1987). The receptor-like protein is encoded by a gene in the VSG gene expression site. Binding of transferrin to the trypanosome transferrin binding protein is however different from binding to the human transferrin receptor. The structures of the two proteins are also different (Schell *et al* 1991a, 1991b). Serum lipoproteins are a further control point in the regulation of growth of trypanosomes. High and Low Density lipoproteins are required for growth of trypanosomes in axenic culture and pose an absolute growth requirement. High and Low Density Lipoproteins are equally effective in promoting growth whilst Very Low Density lipoprotein and Chylomicrons are ineffective (Black and Vandeweerd 1989). This is in apparent variance to High Density Lipoprotein's reputed, although disputed, cytotoxic effect upon *T. b. brucei* in the bloodstream (Lorenz *et al* 1995, Raper *et al* 1996).

Antibody studies have shown trypanosomes to have a receptor homologue for Mammalian Epidermal Growth Factor (EGF) on their cell surface. Trypanosome EGF receptors have associated protein kinase activity which is activated upon occupation of the receptor with

mammalian EGF. The growth of procyclic trypanosomes *in vitro* is enhanced by addition of EGF (Hide *et al* 1989). The concentrations of EGF required for enhancing axenic culture growth of bloodstream trypanosomes (20 - 200nM) were however, very much higher than that occurring in human blood (0.02 - 0.03nM) which would suggest that EGF does not actively influence *T.brucei* proliferation in the mammalian bloodstream. Tissue concentrations of EGF of 120nM do however occur in which case there may be some mitogenic effect upon trypanosomes (Sternberg and McGuigan 1994). This effect may perhaps be critical in the establishment of systemic infection upon injection of small inocula of metacyclic trypanosomes from a tsetse bite.

In contrast to it's inhibitory effects upon the growth of other protozoan parasites, discussed above, IFNγ has a growth promoting effect upon trypanosomes (Olsson *et al* 1991). *In vitro* studies on rat and human MNCs have shown trypanosomes to release a soluble factor, named Trypanosome-derived lymphocyte triggering factor (TLTF), of approximate MW of 185kDa, which causes MNC to release IFNγ. IFNγ, produced by rat MNCs, has in turn been demonstrated to cause an increase in trypanosome proliferation. IFNγ produced by human MNCs, however, does not cause an increase in *T. b. brucei* proliferation (Olsson *et al* 1991). This is a possible mechanism underling the host range specificity of *T. b. brucei*. *T.brucei* induced release of IFNγ has been shown to be $CD8^+$ cell-dependent (Bakhiet *et al* 1990, Olsson *et al* 1991) and more recently the MNC activated by TLTF have recently been shown to be $CD8^+$ cells (Bakhiet *et al* 1993, Olsson *et al* 1993).

Similar to its antiparasitic actions in other species, recombinant TNFα has a growth inhibitory effect upon *Trypanosoma musculi in vitro*. *In vivo*, however and in the presence of peritoneal exudate cells, TNFα enhances parasite growth. It has been theorised that TNFα is directly antitrypanosomal but promotes trypanosome growth through an indirect effect mediated via host cells which may include macrophages (Kongshaun and Ghadirian 1988).

The growth and population density of parasitic infections is a function of both host-derived and parasite-derived factors. Host-derived factors include immune response to the parasite and response of the parasite to host molecules, as discussed above. Parasite-derived processes include internal signals for the parasite to grow, to differentiate and to die. Other factors include the characteristics of antigenic variation of a trypanosome strain i.e. population size of variants, switching rate between variants, variant specific growth rates and the number of variants expressing mosaic VSG molecules (Barry and Turner 1991). A major determinant of the virulence of an infection is the growth rate of the trypanosome population, the higher the growth rate the more virulent the infection. Growth rate is in turn determined by 3 processes; multiplication of slender forms, differentiation from slender to stumpy forms and mortality rates of stumpy forms (Turner *et al* 1995a). Evidence of negative control of growth in chronic infections *in vivo* comes from superimposing a second infection upon a pre-existing infection. The rate of growth in the second infection has been shown to be inhibited (Turner *et al* 1996)

Reduction in growth could be accounted for by a reduction in multiplication rate of slender forms, an increase in rate of differentiation to stumpy forms or by an increase in mortality rate of stumpies. Each could hypothetically be controlled by either internal stimuli or in response to external stimuli. The inhibition described by Turner *et al* (1996) was shown to be at least partly density dependent, suggesting that trypanosomes may have a mechanism of optimising their population numbers without depleting host nutrients or precipitating early onset of pathogenesis and death of the host. It was postulated that the reduction in growth demonstrated in chronic infection is due to a reduction in the growth of slender forms.

Although apoptosis is generally considered to have evolved in order to regulate cell populations in multicellular metazoan organisms recent evidence would suggest the occurrence of apoptosis or Programmed Cell Death, in protozoan parasites. Intracellular parasites appear to influence the apoptosis of their host cells. For example, *Leishmania donovani* is known to prevent the apoptosis of its host macrophages whilst *T.cruzi* induces activation-dependent apoptosis in host infected $CD4^+$ T cells (Welburn *et al* 1997). Apoptosis has also been described in the trypanosomatids *T. brucei rhodesiense* (Welburn *et al* 1997), *T. cruzi* (reviewed by Welburn *et al* 1997) and *Leishmania amazonensis* (Moreira *et al* 1996). These organisms displayed the common ultrastructural and biochemical characteristics associated with programmed cell death in metazoans: destruction of the normal structural organisation of the nucleus due to collapse of chromatin into condensed electron dense masses and fragmentation of parasite DNA into

oligonucleosomal repeat sized fragments. It is a new concept that parasite death, not due to external factors such as toxins or antibodies, may play a central role in the control of the development and course of infection. Apoptosis in *T.cruzi* occurs when proliferating epimastigotes undergo differentiation to the G_0/G_1 arrested stage and is thought to account for the 'stationary phase' of culture. Death can be accelerated or prevented by manipulating culture conditions (reviewed by Welburn *et al* 1997). These data strongly suggest that the trypanosomes are using extracellular signals to regulate their population cell density. Apoptosis is perhaps able to play a role in maintaining genetic stability and population survival of the parasite in the host. The situation of *T.brucei* in the tsetse gut illustrates a possible mechanism by which apoptosis ensures the survival of the parasite population against uncontrolled growth. Once in the tsetse gut trypanosomes endure a long incubation prior to development of a mature infection. During this time trypanosomes are in direct competition with the vector for proline as an energy source. Analysis of trypanosome numbers between 9 and 26 days post infection show parasite numbers to be remarkably constant both within and between flies which is strongly suggestive of a mechanism for self regulation of multiplication and population density. It is highly likely, in view of the existence of apoptosis in culture forms and insect stages of protozoan parasites, that apoptosis exists in mammalian stages and plays a vital role in the control of growth and population density (Welburn *et al* 1997).

1.7 Cell Culture

The *in vitro* culture of cells has been of significant advantage for the study of protozoan parasitology. Culture of both animal and vector stages is possible for most parasites. Leishmania amastigotes and promastigotes, for example, are readily grown in Schneider's Drosophila medium, supplemented with foetal calf serum and gentamycin (Hodgkinson *et al* 1996). For a great many organisms it is now possible to culture the entire life cycle.

Culture of procyclic forms of *T. brucei* preceded that of the bloodstream forms. Early workers in the field cultured procyclic forms on solid nutrient medium and, later, on liquid monophasic or biphasic media containing blood (reviewed by Kaminsky *et al* 1988). Culture of the vector stages of the parasite was achieved in semi-defined media supplemented with foetal bovine serum (Kaminsky *et al* 1988). The most widely used medium for culture of procyclic forms is that of Brun and Schonenberger (1979); SDM-79. It is important to note however that, because of the considerable metabolic differences between bloodstream and procyclic forms, the media used for the culture of the former differ dramatically from SDM-79. The key differences between SDM-79 and the medium used in this project are; a lower concentration of glucose , a higher concentration of sodium-pyruvate, sodium bicarbonate, adenosine, guanosine, and the presence of folic acid and a group of amino acids known to be present in tsetse haemolymph. In addition procylic cultures are maintained at 27°C (Brun and Schonenberger 1979). Such cultures can be established using either bloodstream forms from an infected host or midgut forms

from an infected *Glossina*. Initiation of such cultures is straightforward. The transformation of procyclic to metacyclic forms infective to mice has been described in both axenic cultures (Hirumi *et al* 1992) and in the presence of tissues form the tsetse fly (Cunningham 1986, Kaminsky *et al* 1988) but is not straightforward.

The culture of bloodstream forms of *T.brucei* has been a significant achievement in the study of trypanosome genetics and metabolic studies. Originally trypanosomes were cultured over a layer of feeder cells. This method was introduced by Le Page (1976). Continuous culture of infective monomorphic trypanosomes was achieved later (Hirumi *et al* 1977a; 1977b), followed by culture of infective pleomorphic forms (Brun *et al* 1981). Although solid phases of medium have been developed recently for the culture of trypanosomes (Vassella and Boshart 1996) fluid phase axenic culture offers the most convenient method for investigating the biology of trypanosomes. Semi-defined media allowing the growth of infective forms, with the characteristics of bloodstream forms, in the absence of feeder cells have been described (Baltz *et al* 1985). Variations on this media have been described by other workers (Duszenko *et al* 1985, Sternberg *et al* 1994, Hesse *et al* 1995) and allow the tailoring of growth conditions to meet the requirements of the researcher.

1.8 Aims of this project

In view of the profound effect growth inhibition exerts upon the growth rate of an infection, as demonstrated by Turner *et al* (1996), growth inhibition provides an interesting area for investigation of factors involved in the control of parasite growth. It is of initial interest to attempt to ascertain the origin of a possible signal for growth inhibition. Is the signal of parasite or of host origin? Cytokines, produced by immune cells, have a range of effects modulating the growth of trypanosomes, as has been discussed above. Might the immune cells responsible for such cytokine production be responsible for growth inhibition? Might macrophage-produced NO, a molecule with widely reported effects upon trypanosome survival and growth, be involved also? Attempts to answer these questions were made using the approaches outlined below.

Adoptive transfer experiments using spleen cells were conducted in an attempt to transfer growth inhibition from donor to recipient mice. The role of macrophage-produced NO in growth inhibition was investigated by inducible nitric oxide synthase inhibition studies and by the use of mice genetically manipulated to lack iNO synthase activity. Axenic culture of trypanosomes was also attempted in this project in order to establish a system for investigating the cellular origins of growth inhibition.

CHAPTER 2

AN INVESTIGATION OF THE ABILITY TO TRANSFER INHIBITION OF TRYPANOSOME GROWTH FROM CHRONICALLY-INFECTED MICE TO MICE WITH ACUTE-PHASE INFECTIONS

2.1 Introduction

The growth of trypanosomes in the mammalian host is controlled by factors of both host and parasite origin. Parasite-dependent parameters that may influence the course of infection include the intrinsic growth rate of the strain, the rate of development from dividing to nondividing forms (Turner *et al* 1995a), the spatial heterogeneity of the population within the host, the response of the parasite to host growth factors and, of course, various characteristics of antigenic variation inherent to a particular strain of trypanosome and not modifiable by the host (Barry and Turner 1991). One study, however, has suggested that trypanosome growth rate may be modulated *in vivo*. By superimposing a secondary infection upon a pre-existing infection of a different strain, and using immunofluorescent antibody studies to discriminate between the two infections, the growth of the secondary infection was shown to be greatly inhibited. The later in the primary infection that the second strain of trypanosomes was introduced the more profound the inhibition of growth (Turner *et al* 1996). It remains to be elucidated whether the signal for inhibition of growth is of parasite or of host origin.

One argument suggests that the inhibiting signal is more likely to be of host than of parasite origin. *T.brucei* parasites infect a large range of mammals and the overall levels of parasitaemia differ markedly between species (Aslam 1992). It is difficult to envisage how a density-dependent inhibitory signal could be parasite-derived and yet act at different densities in different host species.

If of host origin, as seems more likely, an inhibitory factor is likely to be a cellular component or product and the most likely candidate cell types are those of reticulo-endothelial origin. The spleen as the primary site of immune priming for a systemic infection would contain populations of cells which had greatest exposure to trypanosomes. A cellular message, causing inhibition of parasite growth may either require cell-cell contact or be in the form of a secreted molecule acting upon the parasite. A postulated secretory molecule may act in paracrine or endocrine fashion. The effect of such a factor acting in an endocrine manner would be demonstrable in blood. In this case, transfer of plasma from an infected to a naive animal may reasonably be expected to result in transfer of growth inhibition. An alternative possibility is that the inhibitory message may act in a paracrine manner or be in the form of a cell surface marker or receptor on host cells and hence would require close host cell to parasite contact. In either of these eventualities it might reasonably be expected that adoptive transfer of spleen cells from chronically-infected to naive animals would cause inhibition of growth of an acute-phase parasitaemia.

I therefore investigated whether there was a possible role for an immune-cell generated factor with an effect upon parasite growth using passive and adoptive transfer assays. To the best of my knowledge adoptive transfer of spleen cells has not previously been used to transfer immunity to trypanosomes from donor to recipient mice. Equally, to my knowledge, this technique has not been used to demonstrate the transfer of growth inhibition. It was the intention of this project to attempt to transfer inhibition of

trypanosomes growth from donor mice to recipient mice using either plasma or a population of spleen cells and to investigate the origin of any factor found to be capable of inducing inhibition of parasite growth.

2.2. Methods

2.2.1. Passive transfer of plasma

The following investigation into the effects of transfer of serum upon trypanosome growth was performed by Nasreen Aslam. Adult female BalbC donor mice were inoculated intraperitoneally with 10^4 EATRO 2340 trypanosomes. On day 20 of infection blood was removed by cardiac puncture into Carter's Balanced Salt Solution (CBSS) containing 10 U/ml heparin. Blood was also obtained from uninfected BalbC mice. Blood was centrifuged at room temperature at 12000g for 2 minutes and plasma collected. Plasma was pooled from batches of infected or uninfected mice, aliquoted and stored at -70°C until required.

Twelve female age-matched BalbC mice were infected intraperitoneally with 10^4 GUTat 7.2 trypanosomes. Twenty four hours later, the 12 mice were split into two groups. One group was inoculated intravenously with 0.5 ml of plasma from the donor mice that had been infected. The other group received 0.5 ml plasma from the uninfected mice as controls.

Parasite densities from both groups were monitored daily between days 1 and 10 post-infection by dilution of peripheral blood, from the tail, in 0.85% ammonium chloride and counting using an Improved Neubauer Haemocytometer.

2.2.2. Adoptive transfers of spleen cells

Spleens were removed from EATRO 2340-infected BalbC mice, on day 19 of infection, under sterile conditions. Spleens from uninfected BalbC mice provided controls. Cells were dissociated from the spleens by repeated percussion with the barrel from a 5 ml syringe through a sterile 'tea strainer' into RPMI 1640 medium (GIBCO) supplemented with 2mM L-glutamine (Sigma), 0.075% sodium bicarbonate (BDH), 2% gentamycin sulphate (Sigma) and 10 % heat inactivated foetal calf serum (GIBCO), and passed through Nirex filters (35µm mesh size; Henry Simon, Stockport England). The resulting single-cell suspension was washed by centrifugation at 300g for 7 minutes at room temperature. The pellet was resuspended in medium and 5 ml aliquots were each layered over 3ml cushions of Nycoprep (1.077g/ml density at 20°C and 265 +/- 5mOsm osmolality, Nycomed) and centrifuged at 1000g for 15 minutes at room temperature. The lymphocytes, which sediment at the interface between medium and Nycoprep, were removed and washed twice by centrifugation at 300g for 7 minutes. A 1 mg/ml stock solution of cymelarsan (Rhone Merieux) was made up with RPMI medium supplemented as described above. Ten µl of the stock cymelarsan solution was added per ml of cell suspension to bring the final concentration of cymelarsan to 10 µg per ml of cell

suspension. This was incubated for 30 minutes at 37°C, 5% CO_2, to kill any trypanosomes contained in the spleen cell preparation. Cymelarsan was washed out of the lymphocyte suspension by centrifuging three times at 300g, room temperature. Splenocyte densities were calculated by counting with an Improved Neubauer Haemocytometer. 1 x 10^7 lymphocytes were inoculated intravenously into each of 12 irradiated (650 Rads) BalbC age-matched female adult recipient mice. Six mice were irradiated but received no splenocytes.

CFLP mice were inoculated intraperitoneally with Glasgow University *Trypanozoon* antigen type 7.2 (GUTat 7.2) trypanosomes (McLintock *et al* 1990) from stabilate diluted in CBSS containing 10 U/ml heparin. When parasites were growing exponentially and consisted mainly of slender forms (at approximately 5 x 10^7 trypanosomes per ml), mice were exsanguinated under halothane anesthesia and parasite densities accurately calculated by counting with an Improved Neubauer Haemocytometer. Twenty four hours after inoculation of lymphocytes, 1x10^4 trypanosomes in CBSS/heparin were inoculated intraperitoneally into each of the 18 recipient mice. The presence of GUTat 7.2 trypanosomes in the blood of recipient mice was confirmed by immunofluorescence of parasites in the subsequent parasitaemia. Indirect immunofluorescence assays were conducted on blood smears fixed in 70% ethanol for 1 hour at 4°C according to the method of Van Miervenne *et al* (1975). Rabbit anti GUTat7.2 antisera was used at a concentration of 1 in 100 in PBS/0.1% Tween 20. FITC labeled anti rabbit antibody (SAPU) was used at a concentration of 1 in 50, in PBS/0.1 % Tween 20.

Parasitaemias were monitored by dilution of 2 µl of peripheral blood (from the tail) in an appropriate volume of 0.85 % NH_4Cl. Samples were taken between days 3 and 8 post infection.

In a repeat of this experiment the irradiated, unreconstituted mice were deemed to be unnecessary and hence were not included in the study.

2.3 Results

2.3.1 Investigation into the effect of passive transfer of serum from infected to uninfected mice.

No significant difference was seen in the parasitaemias of mice which had received serum from chronically infected mice or from control mice (Fig 1). No differences were apparent in rate of log phase growth, in the timing or levels of peak parasitaemia, or in the rates of clearance of trypanosomes.

2.3.2 Investigation into the effect of transferred spleen cells upon parasite growth

Adoptive transfers of splenocytes from infected mice to irradiated recipients followed by infection with *T.brucei*, performed by Nasreen Aslam, showed a trend towards reduced or delayed parasitaemias in recipients of spleen cells from chronically infected donors when compared with controls (Figs 2 and 3). Levels of peak parasitaemia, however, remained unchanged between the two groups of mice.

Two adoptive transfer assays performed by myself gave equivocal results (Figs 4 and 5). One indicated no significant difference between the growth rates of parasites in the recipients of cells from infected donors and in mice which had received no spleen cells (Fig 4). However, a difference was observable between the rates of growth of parasites in recipients of spleen cells from infected mice and from uninfected mice but only at one time point. The second adoptive transfer, in contrast, showed a clear difference between the two groups with lower parasitaemias in mice that received spleen cells from the chronically-infected donors (Fig 5).

2.4 Discussion

The lack of effect of transfer of serum, from infected donors to uninfected recipients, upon parasitaemia and growth rate indicates that no soluble factors in the blood are

responsible for the inhibition of growth demonstrated by Turner *et al* (1996). These data indicate that a secreted molecule acting in endocrine manner is a poor candidate for effecting inhibition. It is possible however that molecules which classically act in paracrine fashion, for example cytokines, might also in some circumstances be released into the systemic circulation. The results from the passive transfer assay suggest that systemic release of such molecules, if it occurs in trypanosome-infected mice, is not responsible for growth inhibition.

The trend towards a reduced rate of growth in the adoptive transfers performed by Nasreen Aslam was not reliably reproducible in the transfers performed by myself. Although peak cell density remained the same, there appeared to be a delay in onset of exponential phase growth. This latter phenotype is the same one described by Turner *et al* (1996). The cause of this trend towards inhibition of growth remains unknown. Killing of parasites before they achieve patency of infection is unlikely as killing could be expected to operate at higher parasite densities and hence result in a lowered level of peak parasitaemia, an effect not evident in these data. Another possible explanation for the delay in onset of exponential growth is the presence of a temporary cytostatic effect effective before exponential growth and which the parasites later overcome. The two phenotypes of growth inhibition and delayed onset of parasitaemia have a similar basis and may appear different only because the assay system used in this study is different from the one used in the Turner *et al* 1996 study. Hence it may be that the delayed onset of growth

observed in recipients of spleen cells from infected donors is evidence of growth inhibition.

Irradiation of recipient mice kills all radiation susceptible immune cells. This would suggest that whilst a factor responsible for slowing parasite growth in the log phase may be present in the radiation resistant immune cell population, no factor is at present capable of reducing peak cell density as seen in the Turner *et al* (1996) study. A factor of lymphoid cell origin influencing parasite growth is further ruled out by the finding that irradiation of mice followed by no reconstitution results in unchanged growth kinetics.

T cells, B cells, neutrophils and NK cells are susceptible to radiation (Abbas *et al* 1994) and would all have been killed when the recipient mice were irradiated. We can therefore discount any effect due to these cell types. Macrophages, as nondividing cells, are radiation resistant at radiation doses of 650 Rads (MacAskill *et al* 1980) and hence any effect upon parasite growth due to macrophages would not have been clearly visible in this assay due to residual macrophages present in the recipient host. The involvement of macrophages in growth inhibition has therefore not been discounted.

The data presented in this study, whilst far from definitive, do suggest that a factor influencing parasite growth is likely to act in paracrine fashion and hence require, if not cell to cell contact, then close spatial relations between host cells and parasites. Cytokines are known to act in this manner and have been shown, in the literature, to have

numerous effects upon trypanosome growth and survival *in vivo* and *in vitro*. Interferon gamma, produced at higher levels in infected animals and cells (Olsson *et al* 1991, Darji *et al* 1993), increases the rate of trypanosome proliferation. Nitric oxide has also been shown to be produced at increased levels during infection (Schleifer and Mansfield 1993, Sternberg and McGuigan 1992, Mabbot and Sternberg 1995) and is an effector molecule of suppressor macrophages (Mills 1991, Sternberg and McGuigan 1992, Schleifer and Mansfield 1993, Mabbott *et al* 1995, Sternberg and Mabbott 1996). In view of NO's effects upon parasitaemia (Sternberg *et al* 1994) it is reasonable to hypothesize that NO may also be in some way involved in growth regulation. Other cytokines produced at higher levels upon infection include IL-1, prostaglandins (Askonas and Bancroft 1984, Sileghem *et al* 1989, Schleifer and Mansfield 1993) and TNFα (Magez *et al* 1993). TNFα has been shown to effect a reduction in parasite growth and an increase in survival time in infected mice (Magez *et al* 1993). Cytokines such as these are produced by immune cells of reticulo-endothelial origin. As a major organ of the reticulo-endothelial system the spleen is likely to contain a large population of cells producing cytokines such as those described. Transfer of growth inhibition with spleen cells, as seen in the transfer assay described in this study, may be attributable to spleen cell production of cytokines with an effect upon parasite growth.

Trypanosomes are covered in a coat of VSG which provides the basis of the parasite's escape of antibody mediated immune response and hence the phenomenon of antigenic variation. In light of the existence of the VSG coat and it's protective properties for the parasite a mechanism of action for a growth inhibitory factor involving cell-cell contact would at first appear unlikely. The VSG coat on trypanosome cell surfaces does not

however preclude the possibility of a cell signaling mechanism such as this. Monoclonal antibodies to mammalian epidermal growth factor have been shown to bind to *T.brucei* membrane fractions (Hide *et al* 1989). In addition epidermal growth factor has been shown to increase the growth rate of trypanosomes *in vitro* (Sternberg and McGuigan 1994). Taken together, the presence of receptors specific for mammalian epidermal growth factor and the mitogenic effect of EGF upon cultured procyclic trypanosomes would certainly suggest that host factors are able to exert an effect upon trypanosomes in the presence of a VSG coat. It remains unknown whether the receptor for EGF is accessible to mammalian blood stream components *in vivo*. A transferrin binding protein has also been identified in membrane fragments of *T.brucei* bloodstream forms. Two facts suggest that expression of the genes for such a protein is linked to expression of the VSG gene. First, the protein is not expressed in the insect-borne stages of the parasites life cycle. Second, although it is unknown how accessible this receptor-like protein is to the external host environment, the protein is encoded by an expression-site-associated gene which is under the control of the promoter transcribing the expressed VSG gene (Schell *et al* 1991a; 1991b). This is suggestive that the protein may be transcribed at the same time as the VSG gene and hence may be expressed with the VSG on the surface of the cell. In light of these discoveries of receptor like proteins, albeit of unknown location, which have been shown to be highly likely to be involved in regulation of growth of the parasite, it is possible that a factor effecting inhibition of growth may act in a manner either involving cell-cell contact or requiring close spatial relations. It is my belief that this may take place without hindrance from a VSG coat.

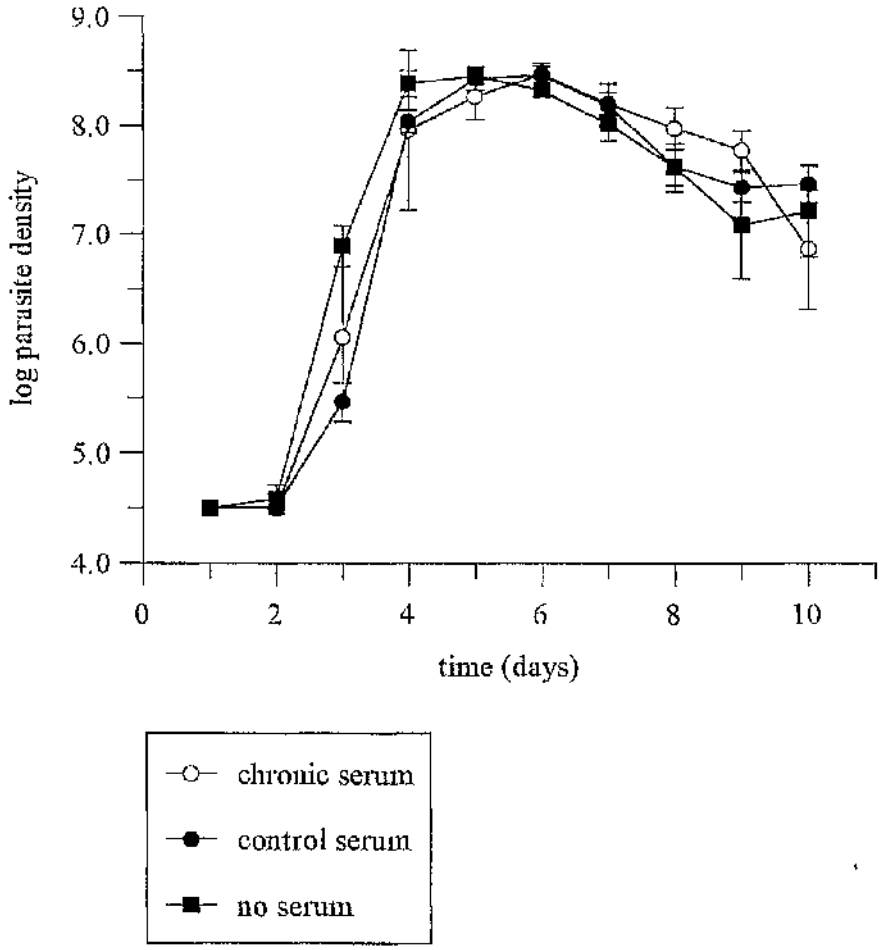


Fig 1. The effect of transfer of serum upon parasite growth.
Passive transfer of serum collected from chronically infected mice and from uninfected controls into trypanosome-infected mice. Data points denote geometric mean +/- 2SE, n = 6.

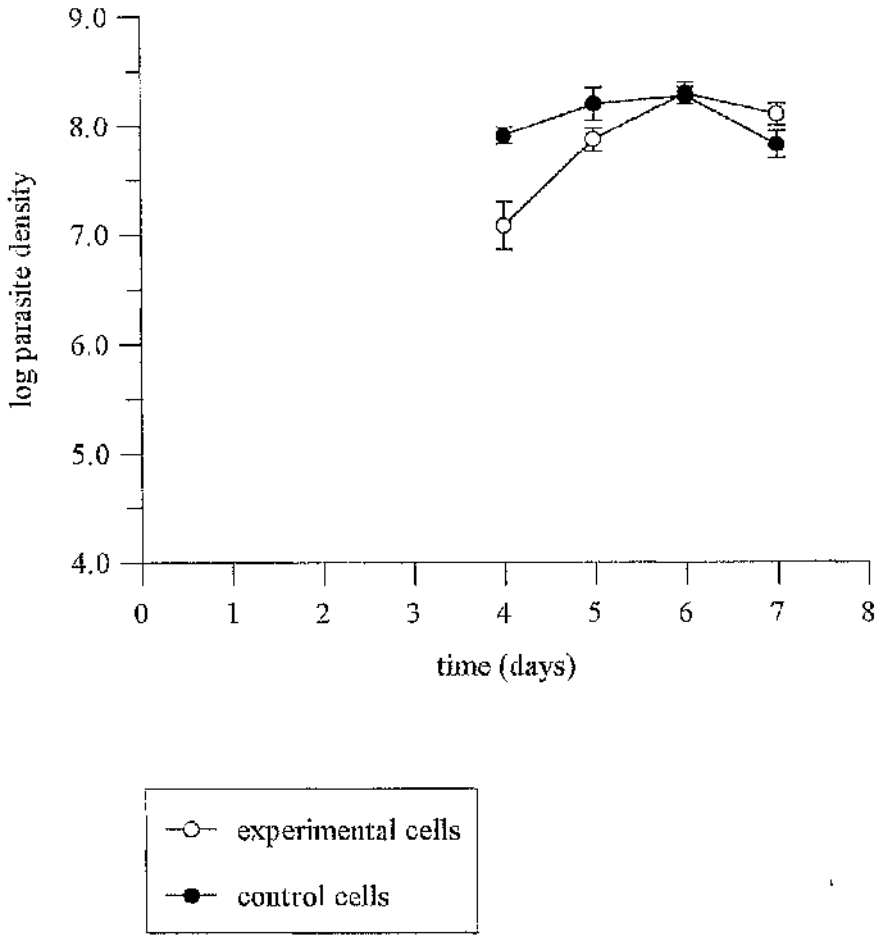


Fig 2. Effect of transferred spleen cells upon parasite growth. No. 1.
Adoptive transfer of 10^7 spleen cells collected from chronically-infected mice and from uninfected control mice. 24 hours after transfer of splenocytes mice recieved 10^4 GUTat 7.2 trypanosomes i.p. Data points denote geometric mean +/- 2SE, n = 6.

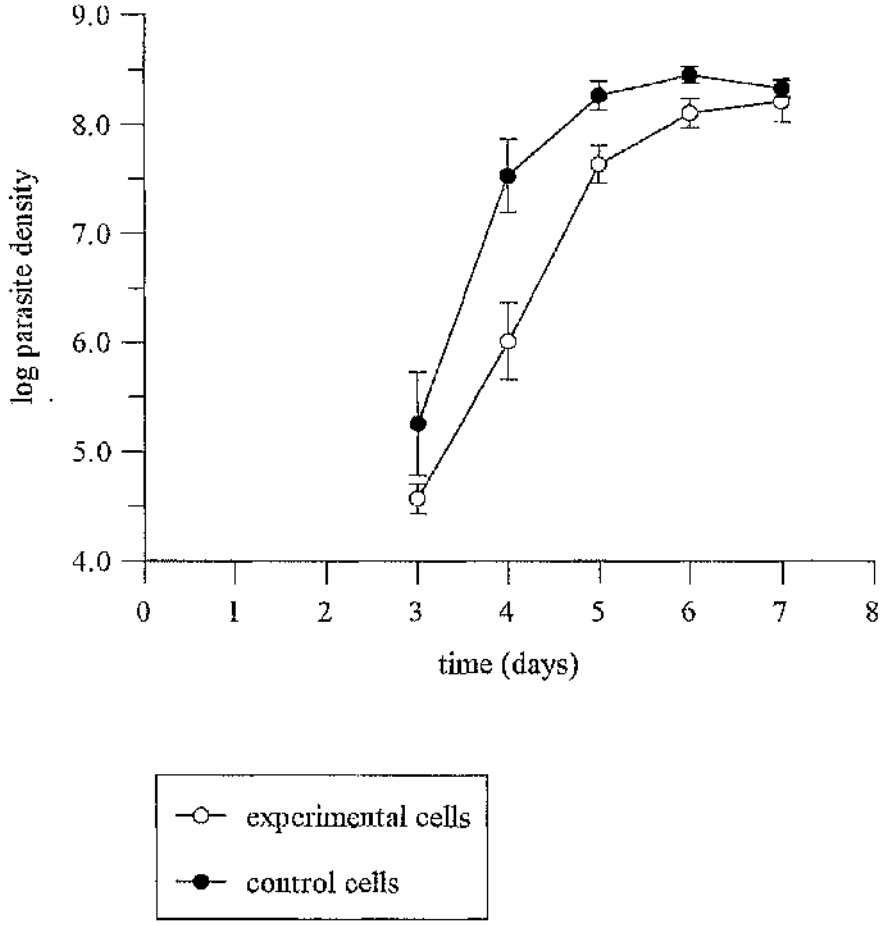


Fig 3. Effect of transferred spleen cells upon parasite growth. No. 2.
Adoptive transfer of 3 x 10^6 spleen cells collected from chronically-infected mice and from uninfected control mice. 24 hours after transfer of splenocytes mice received 10^4 GUTat 7.2 trypanosomes i.p. Data points denote geometric mean +/- 2SE, n = 6.

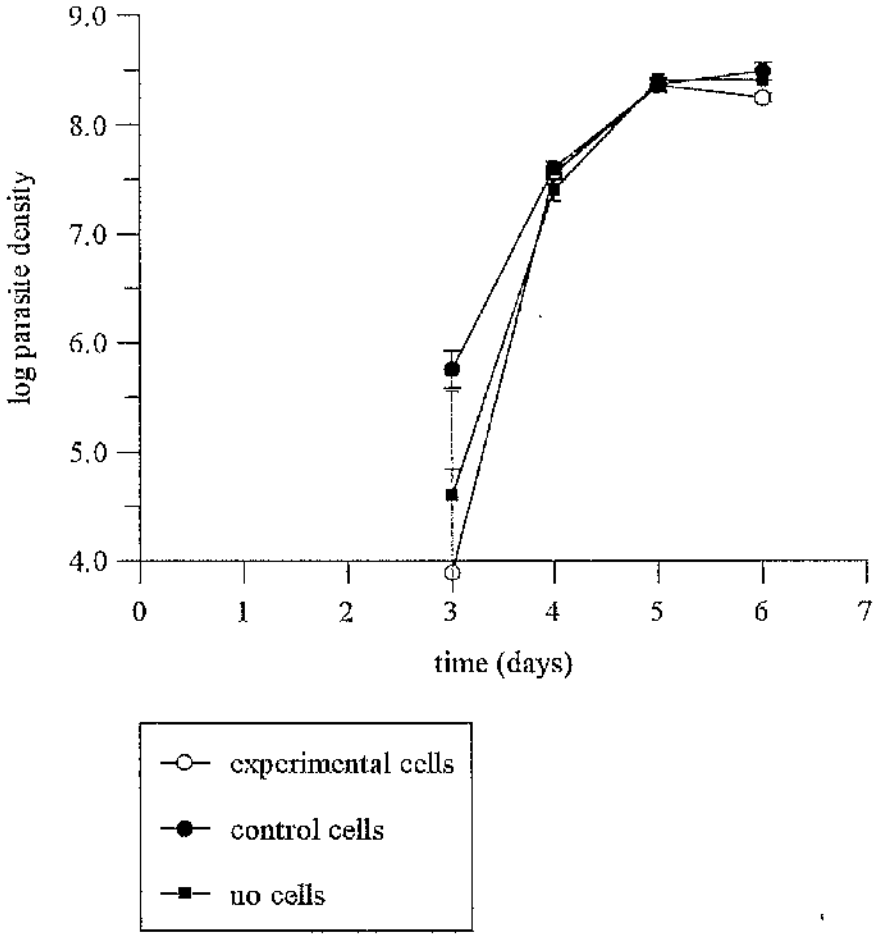


Fig 4. Effect of transferred spleen cells upon parasite growth. No. 3.
Adoptive transfer of 10^7 spleen cells collected from chronically-infected mice and from uninfected control mice. 24 hours after transfer of splenocytes mice received 10^4 GUTat 7.2 trypanosomes i.p. Data points denote geometric mean +/- 2SE, n = 6.

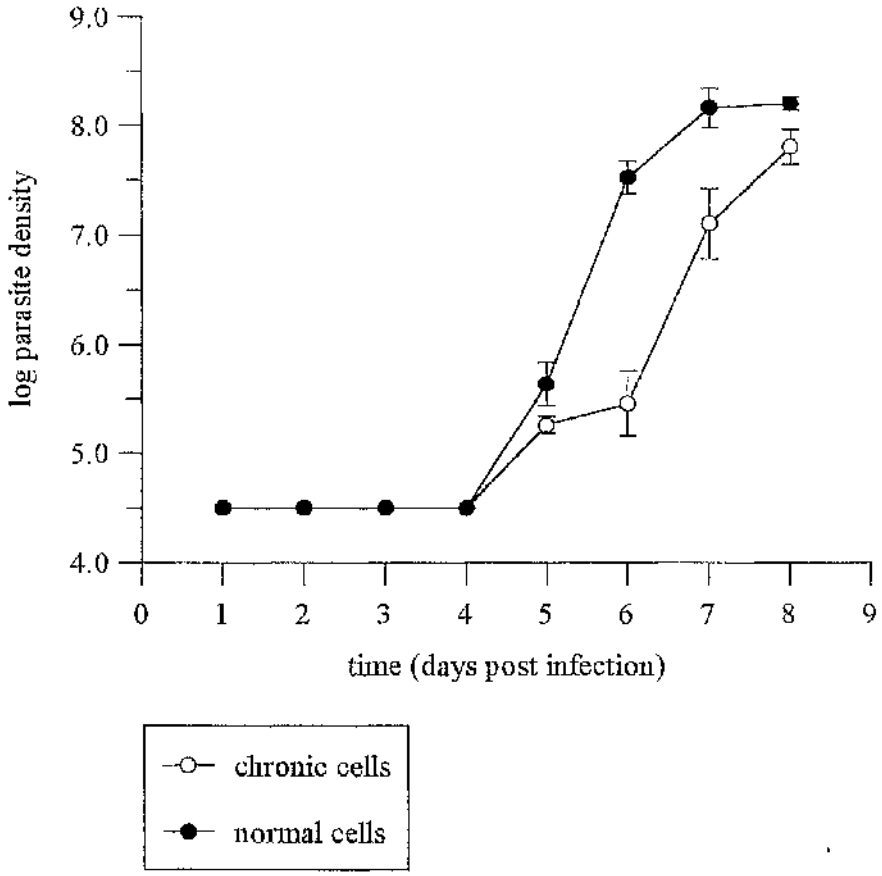


Fig 5. Effect of transferred spleen cells upon parasite growth. No. 4.
Adoptive Transfer of 2 x 10^7 spleen cells collected from chronically infected mice and uninfected controls. 24 hours later recipient mice received 2 x 10^4 GUTat 7.2 trypanosomes i.p. Data points denote geometric mean +/- 2SE, n = 6.

CHAPTER 3

INVESTIGATION INTO THE ROLE OF NITRIC OXIDE IN INHIBITION OF GROWTH OF *TRYPANOSOMA BRUCEI*

3.1 Introduction

Macrophage-produced nitric oxide has been implicated in the suppression of immune responses to African trypanosomes (Mills 1991, Sternberg and McGuigan 1992, Schleifer and Mansfield 1993, Mabbot and Sternberg 1995, Mabbot *et al* 1995). Production of nitric oxide by macrophages and bone marrow cells becomes raised early in murine trypanosome infections (Sternberg and McGuigan 1992, Mabbot and Sternberg 1995) and is thought to mediate suppression of T cell responses (Schleifer and Mansfield 1993, Sternberg and McGuigan 1992). Also, levels of nitric oxide synthase activity are raised in macrophages from infected mice (Mabbot *et al* 1995). It is important to note however that a different situation appears to occur in *Bos indicus*, Boran cattle. Macrophages from infected cattle can be activated *in vitro* to produce nitric oxide metabolites. This response, however, is down-regulated in macrophages isolated from infected cattle. Nitric oxide was found not to mediate T cell suppression in Boran cattle (Taylor *et al* 1996).

A less extensively investigated action of nitric oxide is a potential cytostatic or cytotoxic activity upon *Trypanosoma brucei*. Trypanosomes have been shown to fail to proliferate *in vitro* when treated with nitric oxide gas or exposed to activated macrophages. This effect was reversed when excess iron was provided (Vincendeau and Daulouede 1991, Vincendeau *et al* 1992). This effect of iron is supported by observations that nitric oxide interacts with the Fe-S prosthetic groups in enzymes causing inhibition (Wharton *et al* 1988, Drapier *et al* 1991). The cytostatic effect of activated macrophages has been clearly demonstrated to be due to release of nitric

oxide as established by inhibition studies (Vincendeau and Daulouede 1991). The effect exerted upon bloodstream trypanosomes by nitric oxide has also received recent attention. Inhibition of inducible nitric oxide production *in vivo* by treatment of mice with the substrate analogue N-nitro-L-arginine methyl ester (L-NAME) resulted in a 50% reduction in peak parasitaemia. Paradoxically, addition of the nitric oxide donor, S-nitroso-N-acetyl-penicillamine (SNAP), to axenic cultures of trypanosomes inhibited proliferation by 75%. This effect was also evident in the presence of activated macrophages. Interestingly, addition of whole blood, at dilutions of up to 1 in 200, to these cultures totally abrogated the inhibition of proliferation caused by activated macrophages (Sternberg *et al* 1994). Oxyhaemoglobin is a well characterised scavenger of nitric oxide (Jia *et al* 1996, Lancaster 1994, Wood and Garthwaite 1994) and has been postulated to act as a sink for macrophage-produced nitric oxide hence abrogating any anti-trypanosomal effects of nitric oxide *in vivo*. In this case the reduction of parasitaemia produced by L-NAME administration may be due to removal of the immunosuppressive effects of nitric oxide (Sternberg *et al* 1994) rather than due to a direct cytostatic activity.

Using mixed infections of trypanosomes in mice Turner *et al* (1996) demonstrated the existence of inhibition of trypanosome growth in chronic infections. This phenomenon was postulated to be due to release of a factor of host origin functioning in a parasite-density dependent manner. Inhibition of growth was shown to be exerted upon the entire trypanosome population and not confined to the secondary inocula. Transfers of plasma from infected to uninfected mice followed by infection have failed to illustrate the presence of a soluble host-derived factor acting in an endocrine manner. It would

follow therefore that close host cell to trypanosome contact is required for transfer of a growth inhibitory message.

The involvement of macrophages as producers of such a factor has not been discounted in this project. The extent of the growth regulatory effects of macrophages are likely to be controlled in part by the density of the trypanosomes they come into contact with. Macrophage-produced NO is likely to function in a density dependent, non-specific manner. Due, therefore, to the significant effect of nitric oxide upon the growth and killing of trypanosomes *in vivo* and *in vitro*, the potential role of nitric oxide in inhibition of parasite growth in murine trypanosome infections provides an interesting area for investigation. The available evidence generally suggests that NO could be the factor that modulates parasite growth. The principal rationale against this possibility comes from the scavenging effect of oxyhaemoglobin which would imply that NO could not affect a bloodstream parasite. Whilst this is clearly a strong argument in the systemic circulation, it is not clear to me that it would necessarily hold true for trypanosomes as they traverse the capillaries and venules of the spleen which will be the main site of activated macrophages.

In this chapter I have addressed the question: is NO a modulator of trypanosome growth? I first investigated whether NO was produced during trypanosome infections. Nitric oxide is rapidly oxidised in the blood to nitrite and nitrate, hence levels of nitrite and nitrate were measured as an indication of the levels of nitric oxide produced. I then studied the effect of a reduction in the synthesis of NO upon trypanosome growth in mixed infections using a modification of the chronic infection model developed by Turner *et al* (1996). Reduction in NO synthesis was achieved using two approaches.

Firstly, L-NAME was used to inhibit the production of nitric oxide by inducible nitric oxide synthase (iNOS) *in vivo* by oral administration, in drinking water, to mice as previously described (Sternberg *et al* 1994; Mabbot and Sternberg 1995). NMMA, another widely used substrate analogue inhibitor of iNOS, was not used in these experiments due to financial constraints. In addition, although a large amount of work has been done using NMMA *in vitro* (Vincendeau and Daulouede 1991, Vincendeau *et al* 1992, Green *et al* 1990, Liew *et al* 1990, Mills 1991, Schleifer and Mansfield 1993, Sternberg and Mabbot 1996, Napolitano and Campbell 1994) none has been done in the context of trypanosomiasis *in vivo*. Other novel inhibitors of nitric oxide production (Rajan *et al* 1996) are still at the developmental stage and as yet are not commercially available.

Secondly, transgenic MF1 mice lacking inducible NO synthase activity (Wei *et al* 1995) were used to investigate the role of nitric oxide in growth inhibition.

3.2 Methods

3.2.1. Estimation of the level of nitrite produced in the blood in response to infection with *Trypanosoma brucei*

Two CFLP mice were inoculated intraperitoneally with EATRO 2340 trypanosomes. When trypanosomes were growing exponentially and consisted mainly of slender forms (approximately 10^8 trypanosomes per ml), the mice were exsanguinated under halothane anaesthesia and used as inocula for 6 BalbC age-matched adult female mice.

Blood samples were collected from these mice by venesection into heparinised capillary tubes and plasma collected by centrifugation at 12000g for 2 mins at room temperature in a microhaematocrit centrifuge (Hawksley, England). Samples were obtained before infection and on days 2, 4, 6 and 8 of infection. Plasma samples were stored at -70°C. In a second experiment samples were collected on days 0, 2, 4, 6, 8, 10, 12, 14, 16 and 20 and stored at -70°C.

Parasitaemias were monitored by dilution of 2µl of peripheral blood (from the tail) in an appropriate volume of 0.85% NH_4Cl and counting with an Improved Neubauer Haemocytometer. This was carried out daily from days 1 to 8 post-infection in the first experiment and from days 2 to 20 post infection in the second.

Nitrite contents of plasma samples were estimated by the Griess reaction as described by Ding *et al* (1988). Briefly, the reaction solution was prepared by mixing equal volumes of 0.1% α-naphthyl-amine and 1% sulfanilamide (Sigma). The reaction solution was mixed with an equal volume of plasma, incubated for 10 minutes at room temperature and the absorbance of the end product at 540 nm compared with a standard curve constructed using serial dilutions of sodium nitrite (BDH).

The equation of the Griess reaction is as follows;

$C_6H_4SO_3H + C_{10}H_7NH_2 + NO_2 \Rightarrow C_{10}H_6NH_2N{=}NC_6H_4SO_3H$

(sulfanilamide)(napthyl-amine) (purple end product)

The standard curves were generated by fitting sigmoid curves to the data using "biolynx" software. Sigmoid curves provided the most consistent fit to the data in a number of data sets. The equation of the standard curve in this instance was;

$y = D + (A - D) / (1 + (x / C) \wedge B)$

where: A = 0.002, B = 2.926, C = 25.602, D = 448.725, $R^2 = 0.966$, The standard curve is shown in fig 6.

3.2.2. Estimation of the level of nitrate produced in the blood in response to infection with *Trypanosoma brucei*

Plasma samples were collected as described as above and nitrate present in the samples was reduced to nitrite as described by (Mabbot *et al* 1994). Briefly, nitrate was reduced by nitrate reductase (*Aspergillus* species) (Sigma), in the presence of hydrogen donor NADPH and cofactor Flavin Adenine dinucleotide (FAD) (Sigma). Excess hydrogen was scavenged by addition of lactate dehydrogenase (Sigma) and the cofactor sodium pyruvate (BDH). The reduced samples were assayed by the Griess reaction and absorbances read at 540nm. A standard curve was constructed using serial dilutions of sodium nitrate (BDH). The equation of the standard curve was:

$y = D + (A - D) / (1 - (x / C) \wedge B)$

where A = 0.023, B = 6.003, C = 1.773, D = 0.626, $R^2 = 0.935$

for samples assayed from the acute infection and

A = 0.016, B = 5.060, C = 1.784, D = 0.642, $R^2 = 0.966$

for samples assayed from the more chronic infection. The standard curve for the more chronic infection is shown in fig 7.

3.2.3. Testing of several parameters of the Griess reaction to determine nitrite/nitrate levels in murine plasma

1. Investigation into the presence of endogenous inhibitors to the Griess reaction

The presence of inhibitors to the Griess reaction endogenous to murine blood has been suggested by other workers in the field (Sternberg - personal communication) and would result in reduced detection of nitrite in plasma samples. The presence of endogenous inhibitors was investigated by dilution of plasma samples. Serial dilution of plasma could be expected to dilute the inhibitors to such a levels that they no longer inhibit the Griess reaction. Hence serial doubling dilutions of uninfected murine plasma were prepared with phosphate buffered saline and sodium nitrite was added to each dilution at a concentration of 113 nM. The presence of inhibitors would be indicated by an increase in absorbance at higher dilutions.

Serial dilutions were also prepared of plasma from both control (uninfected) and infected mice in order to ascertain whether dilution of plasma would give accurate estimations of the amount of nitrite and nitrate present in undiluted plasma. Samples were diluted with phosphate buffered saline and the Griess reaction performed as described above.

2. Comparison of the absorbance of sodium nitrite at 540nm and 550nm

Absorbances were read at 540 nm in these experiments rather than 550 nm as described by Ding *et al* (1988), as a matter of convenience and also in order to conserve reagents. The available equipment capable of measuring absorbances at 550 nm required larger sample sizes. In order to ascertain the validity of this approach the absorbances of a serial dilution of sodium nitrite were compared at 550 nm and 540 nm. A serial dilution of sodium nitrite ranging from 0.875 nM to 3620 nM was mixed with equal volumes of the Griess reaction solution as previously described and the absorbance read in duplicate at both 550 nm and 540 nm in a scanning spectrophotometer (Philips) and the standard curves for the two wavelengths were compared.

3. Determination of the stability of the endpoint of the Griess reaction

In the protocol by Ding *et al* (1988) the Griess reaction reaches completion after 10 minutes. For reasons of convenience and due to time constraints it was decided to investigate whether absorbances could accurately be read after more than 10 minutes had elapsed after mixing of samples with the Griess reaction solution.

A standard curve was constructed using concentrations of sodium nitrite ranging from 1.75 nM to 3620 nM. The Griess reaction was performed as previously described and the absorbance read in triplicate at 540 nm after incubation times of 10 mins, 1 hour 40 min, 3 hours, 6 hours and 24 hours. The standard curves obtained at these time points were compared.

3.2.4. The effect of L-NAME administration upon parasitaemia

The activity of the inducible nitric oxide synthase enzyme was inhibited using the substrate analogue Nitro-L-Arginine Methyl Ester (L-NAME, Sigma).
L-NAME was administered in drinking water at a concentration of 1 mg/ml to 6 age-matched female adult BalbC mice. A second group of mice received the D-enantiomer, D-NAME (Sigma), in drinking water and a third group received drinking water with no drug added. Drinking water was provided *ad libitum* and drug solutions were replaced daily.
Two CFLP mice were inoculated intraperitoneally with GUTat 7.2 trypanosomes from stabilate. When trypanosomes were growing exponentially and consisted mainly of slender forms one mouse was exsanguinated into CBSS containing 10 U/ml heparin and the parasitaemia calculated by counting with an Improved Neubauer Haemocytometer. 24 hours after drug administration mice were each inoculated intraperitoneally with 5.8×10^6 trypanosomes. Parasitaemias were monitored approximately every 12 hours as described previously. Mice were euthanased after 134 hours of infection.
In a second experiment, mice were similarly drug treated. Inocula of GUTat7.2 trypanosomes were smaller at 9.6×10^4 trypanosomes per mouse. Parasitaemias were monitored until 166 hours post infection.

3.2.5. Effect of nitric oxide upon parasite growth in mixed infections

3.2.5.1. Effect of administration of L-NAME upon parasite growth in mixed infections

To investigate parasite growth in chronic-phase infections, separately from the confounding effects of antigenic variation and immune responses, an experimental model system was set up, essentially as previously described by Turner *et al* (1996). Trypanosomes of the line EATRO 2340 were raised in two BalbC mice. When trypanosomes were growing exponentially and consisted mainly of slender forms these mice were exsanguinated into CBSS containing 10 U/ml heparin and the blood used to infect intraperitoneally 18 age-matched female adult BalbC mice with 10^6 trypanosomes each. Parasitaemias were monitored daily by the 'rapid matching' technique as described by Herbert and Lumsden (1976).

Nine days post infection the 18 infected mice were split into 3 groups of 6 mice. One group received 1 mg/ml L-NAME in their drinking water, another received 1mg/ml D-NAME in drinking water whilst the third group received water with no addition of drug. Drinking water was provided *ad libitum* and drug solutions were prepared daily. Three days after the drug treatment began, mice were each inoculated intraperitoneally with 10^6 trypanosomes expressing GUTat 7.2 raised from stabilate and growing exponentially in CFLP mice. A fourth group of 6 mice received no drug treatment and was infected with only the second line of trypanosomes, GUTat 7.2.

Parasitaemias were monitored daily as described above. Two blood smears were also prepared from each mouse daily and fixed in 70% ethanol for 1 hour at 4°C. These

were then stored in the presence of silica gel (BDH) at -70°C until required. Mice were euthanased after 22 days of infection.

In mice harbouring mixed infections the relative proportions of the populations of EATRO 2340 and GUTat 7.2 were ascertained by immunofluorescence of the blood smears prepared daily during infection according to a method modified from that described by Van Meirvenne *et al* (1975). Briefly, slides were incubated in PBS/10% FCS for 30 minutes at room temperature and then washed twice in PBS/0.1% Tween 20 (Sigma). Trypanosomes of the line GUTat 7.2 were labeled by incubation with rabbit anti GUTat 7.2 antisera at a concentration of 1 in 100, in PBS/0.1% Tween 20, for 30 minutes followed by washing with PBS/Tween 20. FITC labeled anti rabbit antibody (SAPU) was then used at a concentration of 1 in 50. Trypanosomes were counterstained with 4,6-Diamidino-2-phenylindole (DAPI, Sigma) at a concentration of 0.01 mg/ml. Slides were washed in PBS/Tween 20 and mounted in nonautofluorescing glycerol (Citifluor). 200 parasites were counted per slide and the proportion of GUTat 7.2 present expressed as a percentage of the total parasite density.

Rabbit anti GUTat 7.13 antisera was used at a concentration of 1 in 100, in PBS/0.1% Tween 20, and followed by FITC labeled anti rabbit antibody at a concentration of 1 in 50 as a negative control. A positive control was provided by blood smears prepared from the GUTat 7.2 infected blood used to establish secondary infections, as described above.

This experiment was repeated with the modification that the inocula for both the primary and secondary infections were of 10^4 trypanosomes per mouse.

3.2.5.2. Parasite growth in mixed infections in transgenic mice lacking inducible nitric oxide synthase activity

Trypanosomes of the line EATRO 2340 were raised in two CFLP mice. When trypanosomes were growing exponentially and consisted mainly of slender forms these mice were exsanguinated into CBSS containing 10 U/ml heparin and the blood used to infect intraperitoneally 9 age-matched female MF1 mice transgenic for inducible nitric oxide synthase activity. Five of these mice were homozygous for this mutation, hence were lacking iNO synthase activity, 4 were heterozygous controls. These mice were a kind gift from Professor F. Y. Liew, Department of Immunology, Glasgow University. Each mouse received 10^4 trypanosomes. Parasitaemias were monitored daily by examination of wet blood films. Plasma samples for nitrate and nitrite estimation were obtained from each mouse as previously described before infection and on days 4 and 12 of infection. Nitrate was reduced to nitrite as previously described and assayed by the Griess Reaction.

Standard curves for nitrite and nitrate estimation were in the form;

$y = D + (A - D) / (1 + (x / C) \wedge B)$

where $A = 0.003$, $B = 5.785$, $C = 1.78$, $D = 0.38$, $R^2 = 0.977$

for homozygous mice

and $A = 0.006$, $B = 10.433$, $C = 1.756$, $D = 0.37$, $R^2 = 0.985$

for heterozygous mice.

GUTat 7.2 trypanosomes were raised from stabilate in two CFLP mice. This blood was used to inoculate the MF1 mice. On day 7 of infection mice were inoculated intraperitoneally with 10^4 GUTat 7.2 trypanosomes. Parasitaemias were monitored daily by counting on an Improved Neubauer Haemocytometer as previously described. The proportions which EATRO 2340 and GUTat 7.2 constituted of the overall parasitaemia were calculated as previously described.

This experiment was repeated with the modification that trypanosomes of the line STIB 247 were used as the primary infection. STIB 247 tagged by homologous recombination of a construct conferring resistance to the antibiotic phleomycin onto the tubulin locus (Hope and Turner, unpublished results) were used as the secondary infection. Mice were inoculated with 10^4 trypanosomes of the STIB 247 line intraperitoneally. On day 9 of infection all mice received 10^4 trypanosomes of the line STIB 247 which had been tagged with the phleomycin resistance marker (STIB 247 phleo). Plasma samples were obtained from each mouse before infection and on days 5, 11 and 16 of infection. Nitrite and nitrate content was assayed as previously described.

The standard curve for nitrate estimation was of the form;

$y = D + (A - D) / (1 + (x / C) ^B)$

where $A = 0.003$, $B = 5.785$, $C = 1.78$, $D = 0.38$, $R^2 = 0.977$

for homozygous mice and;

$A = 0.006$, $B = 10.433$, $C = 1.756$, $D = 0.37$, $R^2 = 0.985$

for heterozygous mice.

Blood smears were obtained from each mouse and fixed in a 1:1 acetone:methanol solution at 25°C for 2 - 5 minutes. Proportions of STIB 247 and STIB 247 phleo trypanosomes present were assayed as previously described with the modification that trypanosomes of the line STIB 247 phleo were labeled with rabbit anti-phleomycin antibody at a concentration of 1 in 50 for 2 hours. FITC labeled anti-rabbit antibody was then added at a concentration of 1 in 50 for 30 minutes.

3.2.6. Investigation into the effectiveness of L-NAME at inhibiting production of nitric oxide *in vivo*.

Three groups of 6 BalbC age-matched female adult mice received the following drug treatment; Group 1 received 1mg/ml L-NAME in drinking water, group 2 received 1 mg/ml D-NAME in drinking water and the third group received water with no drug added. Drinking water was provided *ad libitum* and drug solutions were prepared daily.

Trypanosomes from line GUTat7.2 were raised from stabilate in two CFLP mice. When trypanosomes were growing exponentially and consisted mainly of slender forms (at approximately 10^8 trypanosomes per ml), mice were exsanguinated, under halothane anaesthesia, into CBSS containing 10 U/ml heparin. The three groups of drug treated mice were each inoculated intraperitoneally with 10^6 trypanosomes. Parasitaemias were monitored daily by counting with an Improved Neubauer Haemocytometer as previously described. Blood samples were collected in

heparinised capillary tubes by venesection. Plasma samples were pooled for each drug treatment group and stored until required at -70°C.

Nitrite contents in the samples was determined by the Griess assay. Nitrate present in the samples was reduced to nitrite as previously described and the resultant nitrite concentration determined by the Griess assay. The equation for the standard curve was of the form;

$y = D + (A - D) / (1 + (x / C) \wedge B)$

where A = 0.002, B = 2.926, C = 25.602, D = 448.725, $R^2 = 0.966$

for the standard curve constructed with sodium nitrite and

where A = 0.023, B = 6.003, C = 1.773, D = 0.626, $R^2 = 0.935$

for the standard curve constructed with sodium nitrate.

3.2.7. Investigation into the effectiveness of L-NAME at inhibiting nitric oxide production *in vitro*

Macrophages were collected from adult female BalbC mice by peritoneal washes with RPMI 1640 (GIBCO) supplemented with; 2mM L-glutamine (Sigma), 100µg/ml penicillin/streptomycin (GIBCO), 2% of gentamycin sulphate (Sigma), 0.075% sodium bicarbonate (Sigma) and 10% of foetal calf serum (GIBCO).

The cell suspension was washed twice by centrifugation at 250g for 10 minutes at 4°C and cultures established at 2x 10^5 macrophages per well in supplemented RPMI medium in a 96 well culture plate. Macrophages were allowed to adhere to plates for 2 hours at 37°C, 5% CO_2 and then washed by gently pipetting with warm

supplemented RPMI medium to remove non-adherent cells. Media was replaced to restore culture volume to 200µl.

Cells were stimulated with 100ng/ml LipoPolySaccharide (Sigma) and 100 U/ml of recombinant Interferon gamma (a kind gift from Dr. W. Sands, Department of Immunology, University of Glasgow). Inducible nitric oxide synthase activity was inhibited with 1 mM L-NAME. Addition of D-NAME at 1 mM concentration provided a negative control. The adherent cell (macrophage) cultures were incubated at 37°C, 5% CO_2 for 19 hours. The culture supernatant was aspirated and assayed for nitrite content by the Griess reaction.

The equation of the standard curve was of the form;

$y = D + (A - D) / (1 + (x / C) \wedge B)$

where A = 0.021, B = 2.677, C = 19.9, D = 289.597, $R^2 = 0.986$

Nitrate content could not be determined by this method as RPMI contains calcium nitrate at a concentration of 69.49 mg/ml (Johnston and Thorpe 1982).

3.3. Results

3.3.1. Testing of several aspects of the Griess reaction to determine NO_2/NO_3 levels in murine plasma

3.3.1.1. Investigation into the presence of endogenous inhibitors to the Griess reaction in normal murine plasma.

If endogenous inhibitors were present in plasma, dilution of plasma followed by addition of a fixed amount of sodium nitrite would be expected to result in an increase in absorbance as samples become more dilute. As Fig 8 shows, however, as dilution increased absorbance dropped slightly from 0.473 to 0.31 and then remained at this level. These data demonstrate the apparent absence of inhibitors to the Griess reaction in normal murine plasma.

A dilution series of normal mouse plasma was prepared with phosphate buffered saline and absorbance measured at 540 nm in order to ascertain whether dilution of serum with phosphate buffered saline before measurement of absorbance is a valid experimental strategy. Fig 9 show that dilution of plasma leads to a reduced level of absorbance as might be expected. Importantly, however, this relationship is not linear and not robust at higher levels of dilution. For example, dilution by a factor of 4 does not yield 25% of the absorbance. Dilution of plasma samples was not deemed to be a valid experimental strategy. Plasma samples were assayed neat throughout this project.

3.3.1.2. Comparison of absorbance of sodium nitrite at 540nm and 550nm

The standard curves of the Griess reaction product for sodium nitrite when measured at 540 nm and 550 nm were deemed similar enough to justify reading the absorbance of the end product of the Griess reaction at 540 nm throughout this project, Fig 10.

3.3.1.3. Determination of the stability of the colour change of the Griess reaction

Standard curves were constructed using sodium nitrite and the absorbance read at different time points. The curves obtained at 10 minutes, 1 hour and 40 minutes, 3, 6 and 24 hours were almost identical. Hence the colour change produced in the Griess reaction was demonstrated to be stable as shown in Fig 11.

To conclude, nitrite and nitrate levels could be determined in mouse plasma using the Griess reaction. The reaction product of the Griess reaction was stable with time, could be detected at both 550 and 540 nm wavelength and no endogenous inhibitors of the reaction could be detected in plasma. However, dilution of plasma samples before conduct of the Griess reaction, as employed in some other studies (Sternberg - personal communication) gave spurious results.

3.3.2. Determination of the levels of nitrite and nitrate produced in trypanosome infections

The results in Fig 12 show that nitrite production, as measured by the Griess reaction, was detected at low levels, less than 13 nM, before infection and until day 2 post infection. From days 4 to day 6 post infection nitrite was undetectable, then rose to 37.4 nM on day 8 post infection. Nitrate was detected at levels of 20 - 32 nM until day 6 when it rose to 84.3 nM and day 8 when it was detected at 77.5 nM. Levels of nitrate detected in plasma rose significantly shortly after parasitaemia had reached peak levels. These data indicate that trypanosome infections in mice induce NO synthase activity which is trypanosome density-dependent and is detectable after the first parasitaemic peak, mainly in the form of nitrate rather than nitrite.

Fig 13 shows that in chronic as in very acute-phase infections, nitrite was detected at a low level, generally below 20 nM. Nitrate levels rose sharply to 126.4nM at day 8 of infection, dropped again and remained low until day 16 post infection when levels again rose sharply to 171.8 nM. Levels dropped again on day 18 of infection. As was seen in acute-phase infections, production of nitrate was trypanosome density-dependent with peak values approximately 2-4 days after each parasitaemic peak.

3.3.3. The effect of inhibition of nitric oxide synthesis upon parasitaemia

No significant differences were seen between parasitaemias in groups of mice treated with either L-NAME or D-NAME or in the untreated group (Figs 14 and 15), both in

levels of peak parasitaemias and in rates of clearance of infections after peak. In the second experiment (Fig 15) a trend towards reduced rate of growth was seen the L-NAME treated group compared with the untreated group. However the D-NAME treated group showed a greater trend towards reduced rate of growth. These apparent differences between growth rates were not significant.

3.3.4 Investigation into the effect of nitric oxide synthesis inhibition upon mixed infections.

No significant differences were seen in peak parasitaemias between mice treated with L-NAME, D-NAME and in untreated mice (Fig 16A). After day 12 of infection parasitaemias remained high. The secondary infection displayed similar growth kinetics, in mice receiving only the secondary infection, to parasites of the primary infection (Fig 16B). Figs 17A and 17B shown that in the repeat of this experiment the secondary infection again displayed similar growth kinetics to the primary infection. Parasitaemias achieved second peak on day 14 of infection and remained high thereafter.

The growth of the secondary infection, GUTat 7.2, was greatly inhibited in the untreated group of mice (Fig 18). At no point in the infection did GUTat 7.2 constitute more than 2.5 % of the total infection. Growth of GUTat 7.2 was less inhibited in the L-NAME treated group of mice. On day 16 of infection GUTat 7.2 constituted 6.3% of the total infection. This rose to 28.5 % on day 21 post infection

then dropped to 14.3 % on day 22 post infection. The difference in the proportions constituted of the total parasitaemia by the untreated and the L-NAME treated group would suggest a trend towards enhanced growth in the absence of nitric oxide. The error bars however were very large. Also, growth of GUTat 7.2 in the D-NAME treated group was inhibited until day 19 of infection after which GUTat 7.2 constituted 14.5 % of the total parasitaemia on day 21 of infection, and 20.3 % on day 22.

When this experiment was repeated inhibition of the growth of GUTat 7.2 was found to be greatest in the L-NAME treated mice (Fig 19). At no point in the course of infection did GUTat 7.2 constitute more than 5.5 % of the total infection in this group of mice. In the untreated group the proportion GUTat 7.2 constituted of the total parasitaemia rose to 12.5 % on day 14 post infection, dropped again, then rose to 10.4 on day 19 of infection. Error bars were very large and no significant difference was seen between the growth of GUTat 7.2 in the two groups.

3.3.5. Investigation into trypanosome growth inhibition in iNOS-deficient mice

Levels of parasitaemia at first peak were similar in mice both homozygous and heterozygous for the iNOS gene locus (Fig 20). In the homozygous group of mice parasitaemias were resolved more rapidly after the first peak of infection and reached lower levels on days 7-10 than in the heterozygous group. The heterozygous group exhibited parasitaemias which remained high throughout the course of the experiment. After the first peak of parasite density had been passed error bars became very large as

has been observed previously with this line of parasites (Turner *et al* 1995). For this reason it is informative to examine the parasitaemias in individual mice (Figs 21 and 22) as well as the mean values for each group. These data show that there was considerable variability between individual mice after the first parasitaemic peak. For example, some mice exhibited three peaks of parasitaemia whilst in other animals only one peak was observed. Addition of secondary infections to mice had negligible effect upon parasitaemias, as would be expected given that a second inoculum of 10^4 trypanosomes was introduced to a pre-existing infection of roughly 10^7 parasites per ml. In a formal comparison no differences in parasitaemia were seen (Turner - personal communication).

Growth of the secondary infection, GUTat 7.2, was greatly inhibited in both groups of mice (Fig 23). At no point did GUTat 7.2 trypanosomes constitute more than 15% of the total parasitaemia. No differences in levels of GUTat 7.2 between the two groups were significant.

Plasma from mice heterozygous for the iNOS gene locus contained summed nitrite and nitrate at levels between 31.7nM and 38.2nM on day 4 of infection, Table 1. Levels rose to between 37.4nM and 50.5nM on day 8 of infection. Mean values rose over this time from 34.8nM to 48.0nM. Plasma from mice homozygous for the iNOS gene locus displayed greater variability. Nitric oxide metabolite was detected at levels between 24.9nM and 52.6nM on day 4 of infection. Plasma from two mice contained summed levels of nitrite and nitrate of below the nM range. Levels of nitric oxide

metabolite on day 8 of infection were between 24.2nM and 83.1nM. Mean values of nitrite and nitrate rose during this time from 21nM to 55.2nM.

A repeat of this experiment was conducted using a different cloned line of parasite (STIB 247) that produces lower parasitaemias and is less virulent for mice permitting infection to be followed over a longer time period. BalbC mice, for example, infected with EATRO 2340 develop unacceptable symptoms of disease after approximately 20 days of infection but can readily tolerate infections with STIB 247 for over 30 days (Turner *et al* 1995). In this experiment all mice, whether homozygous or heterozygous, reached first peak at the same time. Parasitaemias in homozygous mice dropped faster after the first peak of parasitaemia and reached a lower level than in the heterozygous mice (Fig 24). Interestingly, parasitaemias in the homozygous mice reached second peak sooner than in the heterozygous mice. Considerable variability was evident between individual mice after the first parasitaemic peak Some mice exhibited two peaks of parasite density whilst others exhibited three (Figs 25 and 26). It had been the intention to discriminate between parasites in the primary and secondary infections and thus investigate parasite growth in the context of a chronic infection using the phleomycin resistance markers as an epitope tag that would be detected using an antibody by immunofluorescence. However, due to very high levels of non-specific binding experienced with the anti-phleomycin antibody, it was not possible to calculate the proportion of the total parasitaemia constituted by the transfected STIB 247 line. Attempts to over-come this problem, including manipulation of antibody concentrations, incubation times and washing conditions, were unsuccessful.

In contrast to the previous experiment all the mice homozygous for the iNOS gene locus displayed a surprising and inexplicable reduction in summed levels of nitrite and nitrate over the course of infection. Table 2 shows mean values which dropped over the course of infection from 37.5uM in control, uninfected, plasma to 24.0uM on day 16 of infection. One data point, which was over the uM range, was discarded due to suspected haemolysis in the plasma sample resulting in erroneous values. Three out of the four mice heterozygous for the iNOS gene locus displayed levels of nitrate and nitrite which dropped over the course of infection. One mouse however exhibited levels that increased slightly (Table 2). Mean values of nitric oxide metabolite rose transiently from 40.4uM in control uninfected plasma to 42.9uM on day 5 of infection. Levels then fell to 34.8uM and 36.7uM on days 11 and 16 of infection respectively.

3.3.6 Effectiveness of L-NAME in inhibition of nitric oxide synthase

The contrast between the results for parasitaemias when using L-NAME as an inhibitor and with iNOS-deficient mice would suggest that the inhibition is ineffective, even though this has been successfully used in other studies. In this study, administration of L-NAME would appear to have had no inhibiting effect upon the production of nitric oxide *in vivo*. Levels of nitrite produced in the plasma of L-NAME treated mice were higher than or equal to the levels produced in the plasma of D-NAME treated mice and in untreated mice at all points tested, (Fig 27). Levels of nitrate detected in the plasma of L-NAME treated mice were slightly lower than in either untreated mice or in mice which had received D-NAME (Fig 28). However production of nitrate was not abrogated suggesting that enzyme activity was still present.

To test further the efficacy of L-NAME, I conducted an experiment *in vitro*. Addition of L-NAME to cultures of stimulated peritoneal macrophages resulted in a 35 % reduction in the production of nitrite in the culture supernatant compared with cultures without the addition of L-NAME (Fig 29).

3.4. Discussion

Before investigating the potential role of nitric oxide (NO) in trypanosome growth, preliminary experiments were conducted in order to optimise the Griess assay, establish levels of NO produced in BalbC mice and to reproduce observations made by other workers in the field on the effect of inhibition of inducible nitric oxide synthase upon parasitaemias in mice of a different genetic background.

Nitrate production has, in accordance with previous observations, been shown to increase during infection. In this study nitrate levels were found to rise significantly above baseline levels after 5 to 6 days of infection. This result compares favourably with the *in vitro* production of NO by peritoneal macrophages 3 days post infection (Sternberg and McGuigan 1992) and increased levels of nitrate detected in mouse urine at day 3 post infection (Sternberg *et al* 1994). Nitrite levels failed to rise above baseline levels during the course of infection. As nitrite rapidly oxidises to nitrate in the blood, measurement of nitrate gives a more accurate indication of levels of NO produced. In the limited time course of this infection, levels of nitrate rose after parasitaemia reached peak density suggesting that parasite density is likely to have a role as a causative factor in increased production of NO.

In contrast with previous studies (Sternberg *et al* 1994) inhibition of inducible NO synthase had no significant effect upon parasitaemia. The difference between the levels of nitrite and nitrate detected in the plasma of untreated and L-NAME treated mice in response to infection in this lab and that reported by other workers (Sternberg - personal communication) could be due to the different breeds of mouse used having different sensitivities to L-NAME. Alternatively, levels of nitrate and nitrite in plasma were detected by Sternberg after dilution of plasma samples with phosphate buffered saline to dilute out putative inhibitors of the Griess reaction endogenous to murine plasma. This was a strategy found to be invalid in my hands after a consistent failure to demonstrate the presence of inhibitors endogenous to murine plasma. The possibility may exist therefore that the levels of NO metabolites detected by Sternberg (Sternberg and McGuigan 1992, Mabbot and Sternberg 1995) were artificially raised by conducting all Griess assays on diluted plasma samples.

The lack of effectiveness of L-NAME is further illustrated by the levels of nitrite produced by activated peritoneal macrophages, in the presence of L-NAME *in vitro*.

Another possible explanation for the apparent lack of effect of L-NAME upon parasitaemia in the current study may be the difference in genetic background of both the mice and the trypanosome lines used in the afore-mentioned study. In the present study, inbred female BalbC mice were infected with GUTat 7.2 trypanosomes whilst in the former study male C3H.He mice were infected with AnTat 1.1 trypanosomes. Whilst the sexes of the mice has a small effect on parasitaemia (Greenblatt and Rosenstreich 1984), the strain of trypanosome and the genetic background of the mice is likely to have a far greater impact (Levine and Mansfield 1981, Turner *et al* 1995). In a more susceptible breed of mouse the presence or absence of a factor influencing

growth of the parasite, i.e. NO, could be expected to have a greater effect upon parasitaemia than it would in a breed of mouse displaying a greater degree of resistance to infection. In this case the presence or absence of NO could be expected to be more likely to have an effect upon parasitaemia in the more susceptible C3H.He than in the relatively resistant BalbC mice.

The inhibition of growth seen in the secondary infection in the untreated or the D-NAME treated mice is in keeping with previous observations (Turner *et al* 1996). The removal of such inhibition in the presence of L-NAME in the first experiment is suggestive of a role for NO in growth inhibition. However this result was not robust in that it was not observed in the repeat experiment.

Upon infection of mice deficient in iNOS the lack of difference in parasitaemias between mice homozygous and heterozygous for the iNOS gene locus would at first sight suggest that NO has little effect upon parasite growth when these strains of trypanosome are grown in mice with the MF1 genetic background. The rate of clearance of parasites from the systemic circulation does, however, appear to be increased in the case of mice homozygous for the iNOS gene locus. In view of the immunosuppressive activities of NO reported in the literature, (Mills 1991, Sternberg and McGuigan 1992, Schleifer and Mansfield 1993, Mabbot and Sternberg 1995, Mabbot *et al* 1995), a possible role for NO in the mice heterozygous for the iNOS gene locus might include exerting an immunosuppressive action upon the host, thereby lessening the rate of clearance of the parasites from the blood. As T cell help is required for the production of specific antibodies by B cells, suppression of T cell help

might reasonably be expected to result in reduced antibody production and hence reduced clearance of parasites.

As no difference in the level of the growth inhibition of the second infection was observed between the two groups of mice it would appear that NO has no role as the putative factor responsible for growth inhibition.

We can attribute the large error bars in both overall parasitaemias and in levels of the secondary infection to the outbred genetic background of MF1 mice.

Throughout this series of experiments the existence of growth inhibition has been reliably demonstrated in a variety of combinations of mouse and trypanosome strains. A role for NO in control of parasite growth has been strongly implicated by other workers (Vincendeau and Daulouede 1991, Vincendeau *et al* 1992, Sternberg *et al* 1994, Albright *et al* 1994, Mabbot *et al* 1994). My results do not support this and would suggest NO to have little or no effect upon trypanosome growth kinetics. The lack of effectiveness of L-NAME and the evidence of significant levels of NO production in mice lacking the iNOS gene locus would go a long way to explaining the above discrepancy. Production of NO by mice lacking the iNOS gene locus demonstrated in this lab, both here and in a separate study (Millar and Turner - unpublished results) has two possible explanations. Firstly, the disruption of the locus for iNOS may be incomplete in these mice and some residual enzymatic activity may still remain. Alternatively, as has been suggested by other groups (Kantor *et al* 1996), animals lacking enzymatic activity for one isoform of nitric oxide synthase are able to utilise other isoforms in their place. Although I have been unable to find evidence in

the literature of this occurring in mice lacking inducible NOS activity it would be unwise to discount this possibility. The occurrence of upregulation of other isoforms of NOS in the mice used in these studies would explain the levels of nitric oxide metabolite detected in mice with iNOS either inhibited or functionally absent.

Haemoglobin has been demonstrated by other workers in the field to abrogate the antitrypanosomal effects of NO *in vitro* (Mabbot *et al* 1994, Sternberg *et al* 1994). Oxyhaemoglobin's role as a scavenger of NO is well documented (Goretski and Hollocher 1988, Lancaster 1994, Wood and Garthwaite 1994) and is postulated to limit NO's sphere of action. Inactivation of NO has, however, been argued to have little effect upon NO's sphere of influence due to its fast rate of diffusion. The physiological sphere of influence of a single point source of NO that emits for 1 - 10 seconds has a diameter of about 200μm (Wood and Garthwaite 1994) which, in the context of a capillary bed, is a considerable distance. It has been further suggested that NO is able to exert its biological effects, in the presence of high levels of oxyhaemoglobin, by endogenous formation of s-nitrosothiols which retain nitric oxide-like vasorelaxant activities but are not subject to the diffusioual constraints exerted upon NO by oxyhaemoglobin. Arterial-venous differences in the s-nitrosothiol content of intra-erythrocytic haemoglobin suggest that once NO has been scavenged by oxyhaemoglobin it is released during the arterial-venous transit. Nitric oxide then reacts with endogenous thiol groups in the blood to form s-nitrosotiols. This would suggest that in the absence of free NO, nitroxy-haemoglobin can deliver NO activity in a manner not scavenged by haemoglobin (Jia *et al* 1996). It would appear probable that if s-nitrosothiols retain NO-like vasoactive properties that their presence may

explain the effects observed upon parasite growth in the blood of infected animals as observed by other workers (Sternberg *et al* 1994 and Mabbot *et al* 1994).

In conclusion, I have confirmed the results from other groups demonstrating that trypanosome infections cause a rise in nitric oxide production. I have then extended our understanding of how NO acts in trypanosomes infections in two important respects. Firstly, NO does not affect the growth of trypanosomes in either acute- or chronic-phase infections. Although this result was predicted from previous work (Mabbot *et al* 1994) the rationale on which that study was based was limited in three respects. It overlooked the requirement for trypanosomes to traverse the splenic capillary bed when macrophages may have close contact with parasites in the effective absence of haemoglobin, it pre-empted description of the potential formation of nitrosothiols and neglected the high rate of diffusion of NO. Secondly, I have presented data which suggest that L-NAME is only a poor iNOS inhibitor. As a result data published using that inhibitor may need reinterpretation. Nevertheless, results from using iNOS-deficient mice suggest that NO does have an effect on parasitaemia but this effect is likely to be mediated indirectly through modulation of the effector immune response.

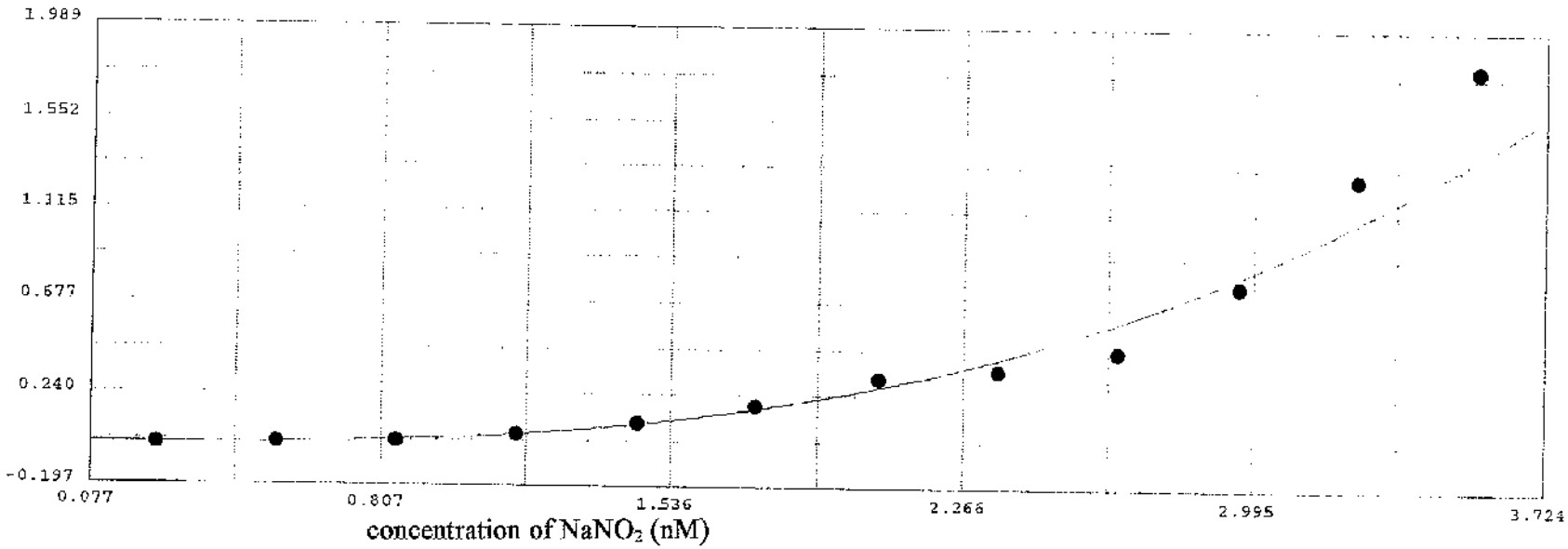


Fig 6. An example standard curve for the Griess assay, used to detect sodium nitrite.
A dilution series of sodium nitrite was constructed and subjected to the Griess assay with PBS as negative control. Absorbances were read at 540 nm. Data points denote geometric mean, n = 3.
Equation; $y = D + (A - D) / (1 + (x / C)^B)$ where A = 0.002, B = 2.926, C = 25.602, D = 448.725, $R^2 = 0.966$.

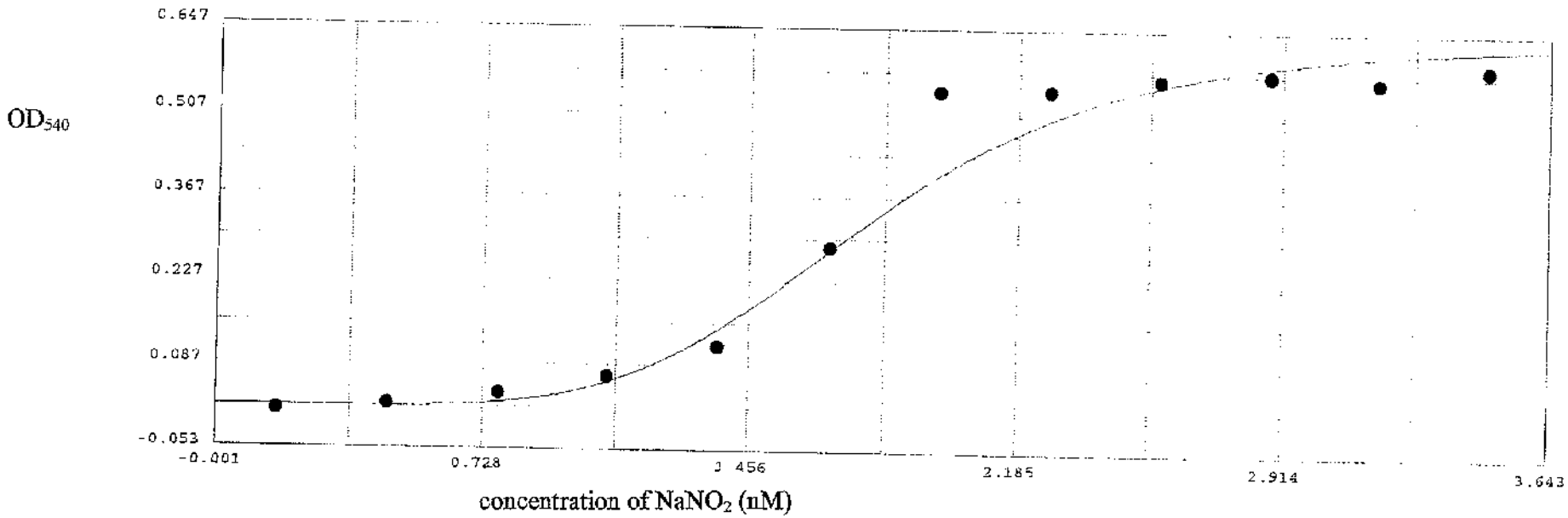


Fig 7. An example standard curve for the Griess assay, used to detect sodium nitrate.
A dilution series of sodium nitrate was constructed and subjected to the Griess assay with PBS as negative control. Absorbances were read at 540 nm. Data points denote geometric mean, n = 3.
Equation; $y = D + (A - D) / (1 + (x / C)^B)$ where A = 0.016, B = 5.060, C = 1.784, D = 0.642, $R^2 = 0.966$

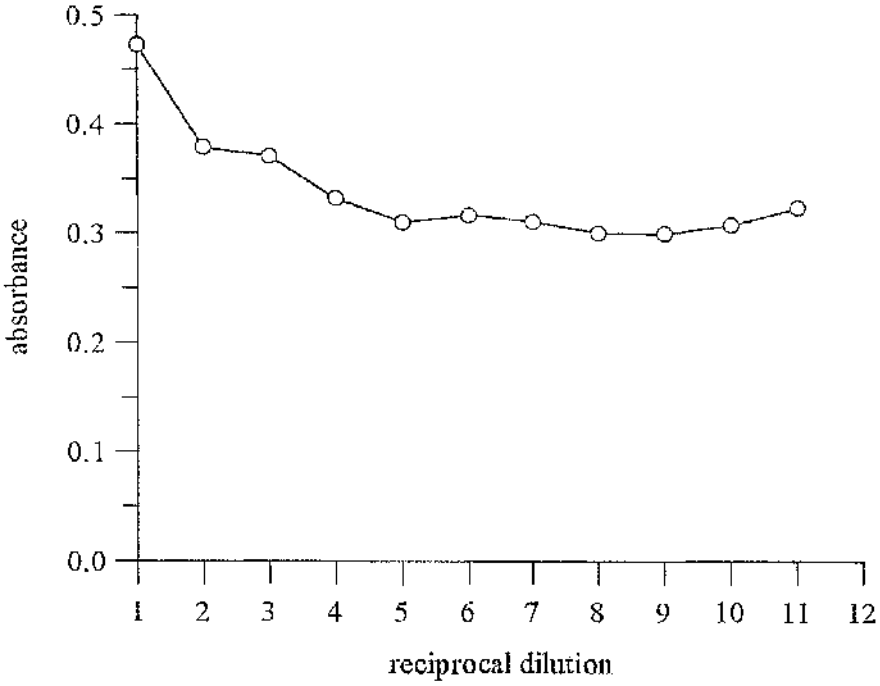


Fig 8. Apparent absence of inhibitors of the Griess reaction in normal mouse plasma.
A dilution series of normal (uninfected) murine plasma was prepared with phosphate buffered saline. Sodium nitrite was added to each sample which was then subjected to the Griess reaction and absorbance read at 540 nm.

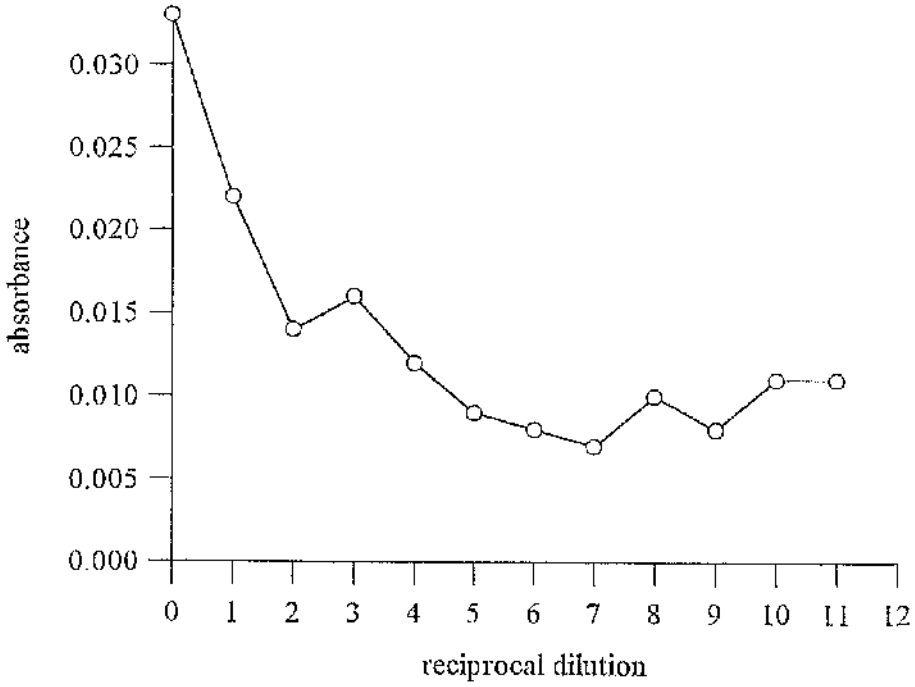


Fig 9. Effect of dilution of murine plasma upon absorbance.
A dilution series of uninfected murine plasma was prepared with phosphate buffered saline. Samples were subjected to the Griess reaction and absorbance read at 540 nm.

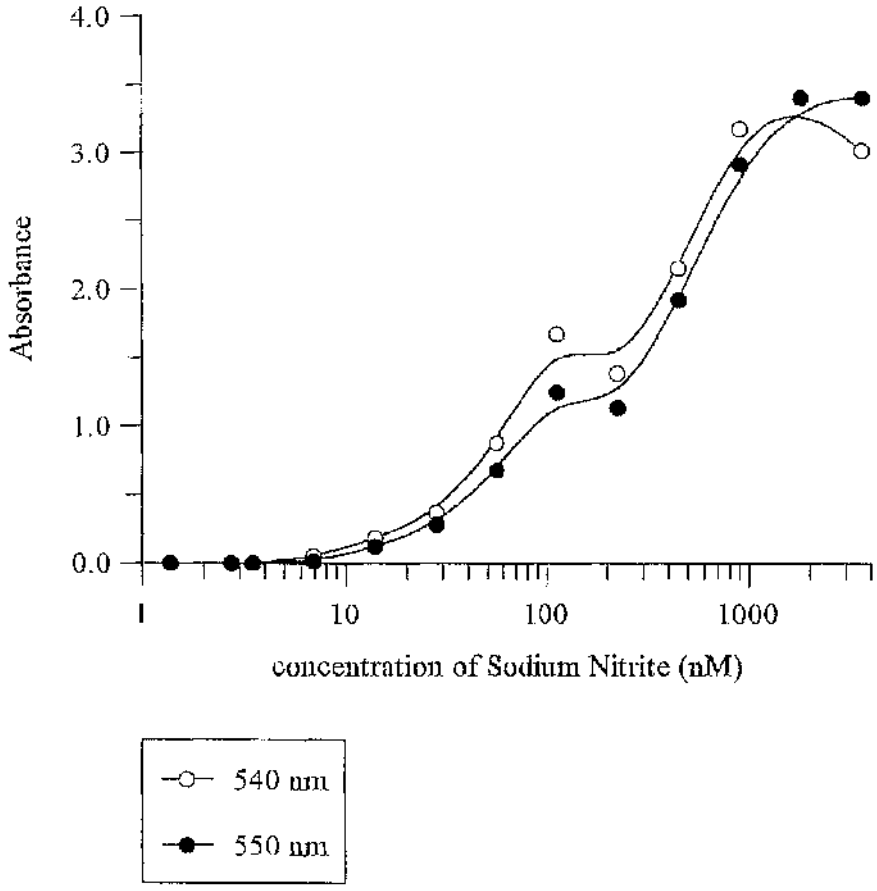


Fig 10. Comparison of standard curves of the Griess reaction product for sodium nitrite when measured at 540 nm and 550 nm.
Dilution series of sodium nitrite were prepared with phosphate buffered saline. Griess reaction products were then read at 540 nm and 550 nm. Data points denote average values, n = 2 and the lineswere fitted using a B-spline algorithm in GRAFIT software

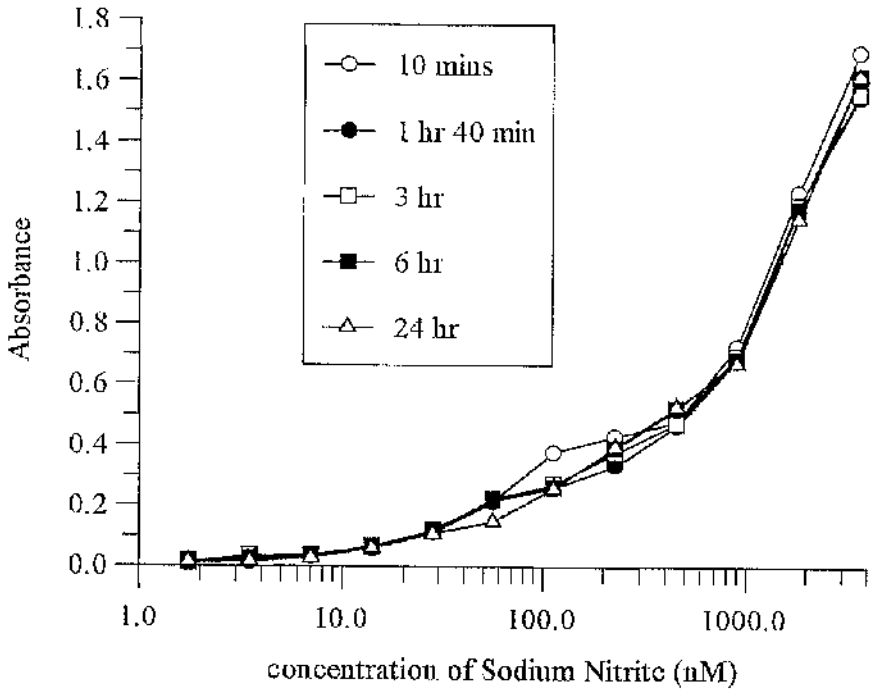


Fig 11. Stability of the colour change in the Griess reaction.
A dilution series of sodium nitrite was prepared with phosphate buffered saline. Absorbance was read at 540 nm, after 10 minutes, 1 hour and 40 minutes, 3 hours, 6 hours and 24 hours.

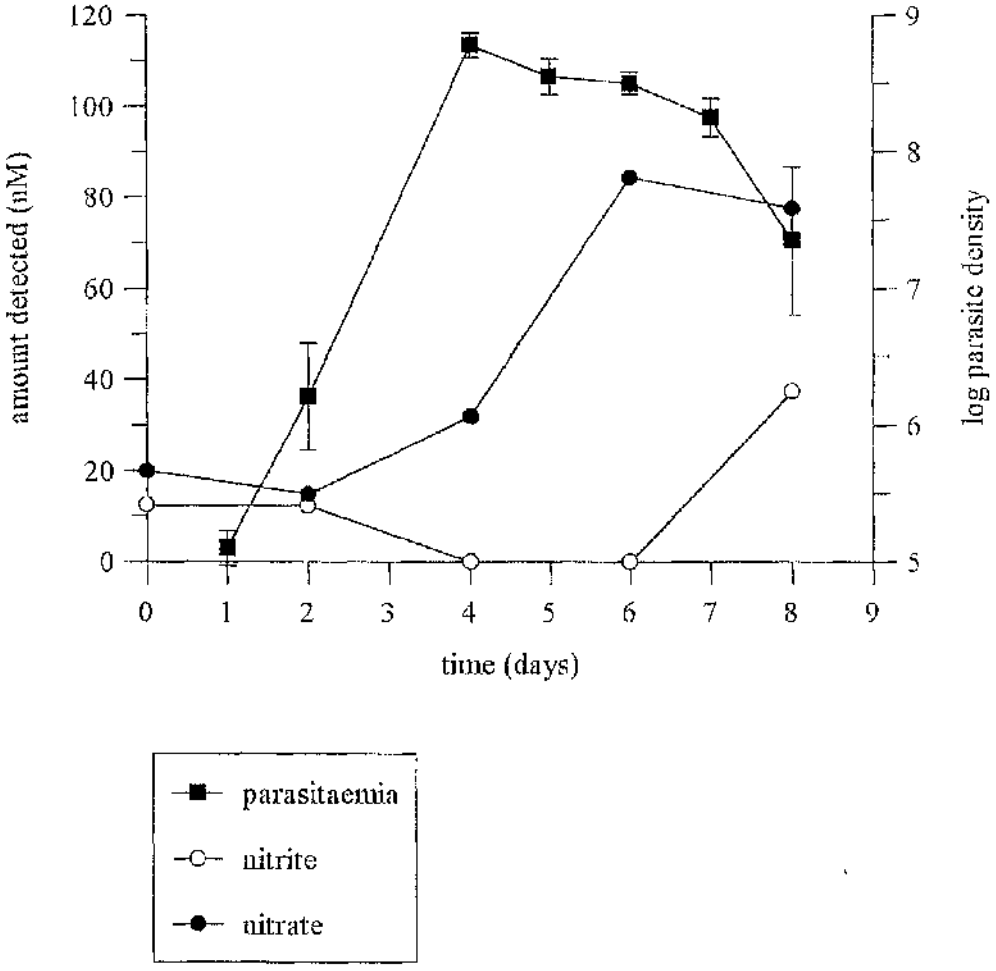


Fig 12. Levels of nitrite and nitrate in plasma during acute-phase trypanosome infections.
Plasma samples were obtained from EATRO 2340-infected mice. Nitrate was reduced to nitrite prior to the Griess reaction.

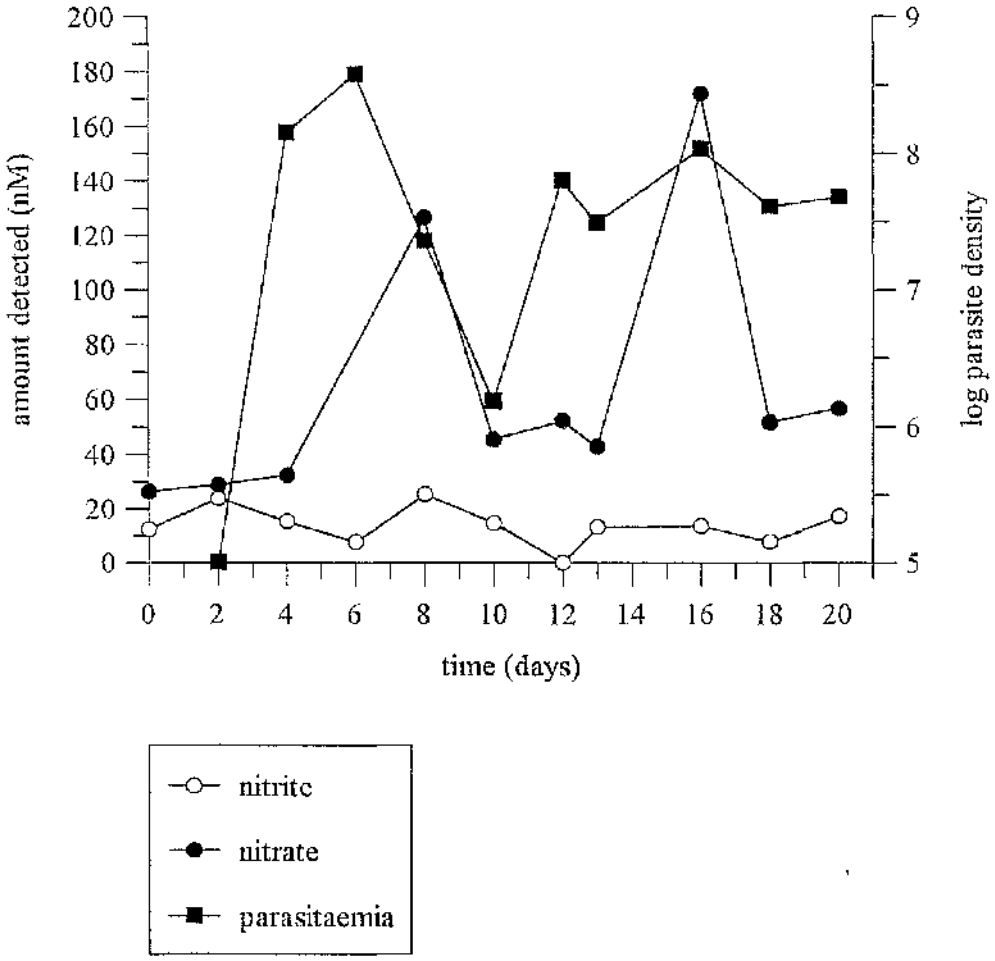


Fig 13. Levels of nitrite and nitrate in plasma during chronic-phase trypanosome infection.
Plasma samples were obtained from EATRO 2340-infected mice. Nitrate was reduced to nitrite prior to the Griess reaction.

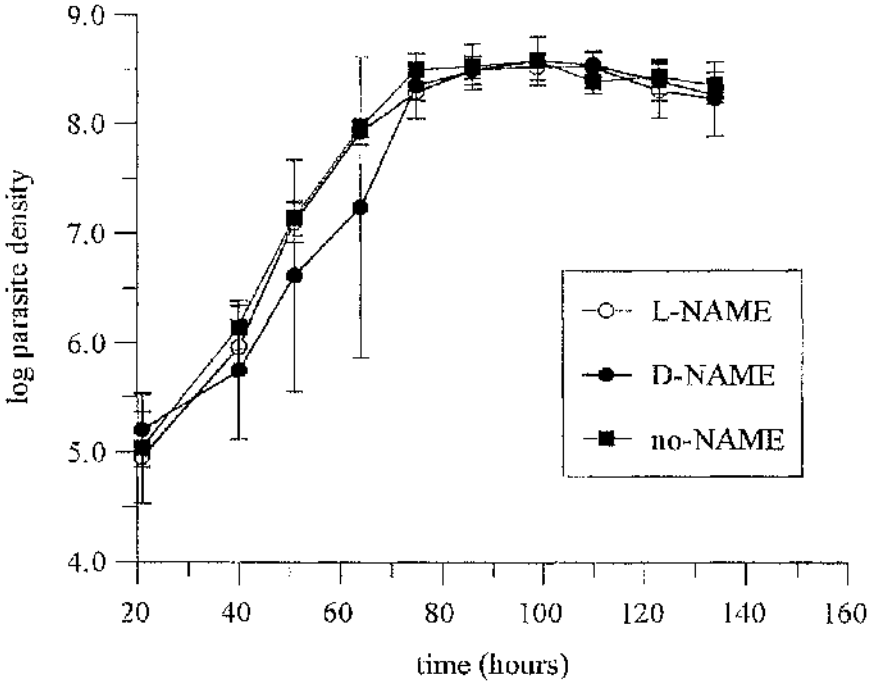


Fig 14. Parasitaemias in three groups of mice after administration of NO synthase inhibitors. Course of infection in mice receiving the NO synthase inhibitor, L-NAME, and in control groups. Data points denote geometric mean +/- 2SE, n = 6.

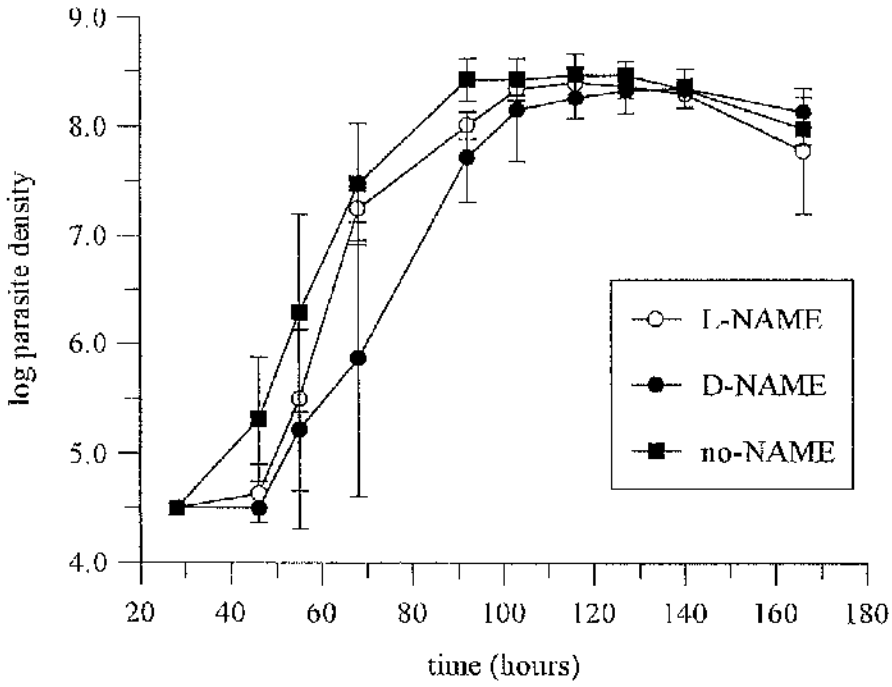


Fig 15. Parasitaemias in three groups of mice after administration of NO synthase inhibitors
Course of infection in mice receiving the NO synthase inhibitor, L-NAME, and in control groups. Data points denote geometric mean +/- 2SE, n = 6.

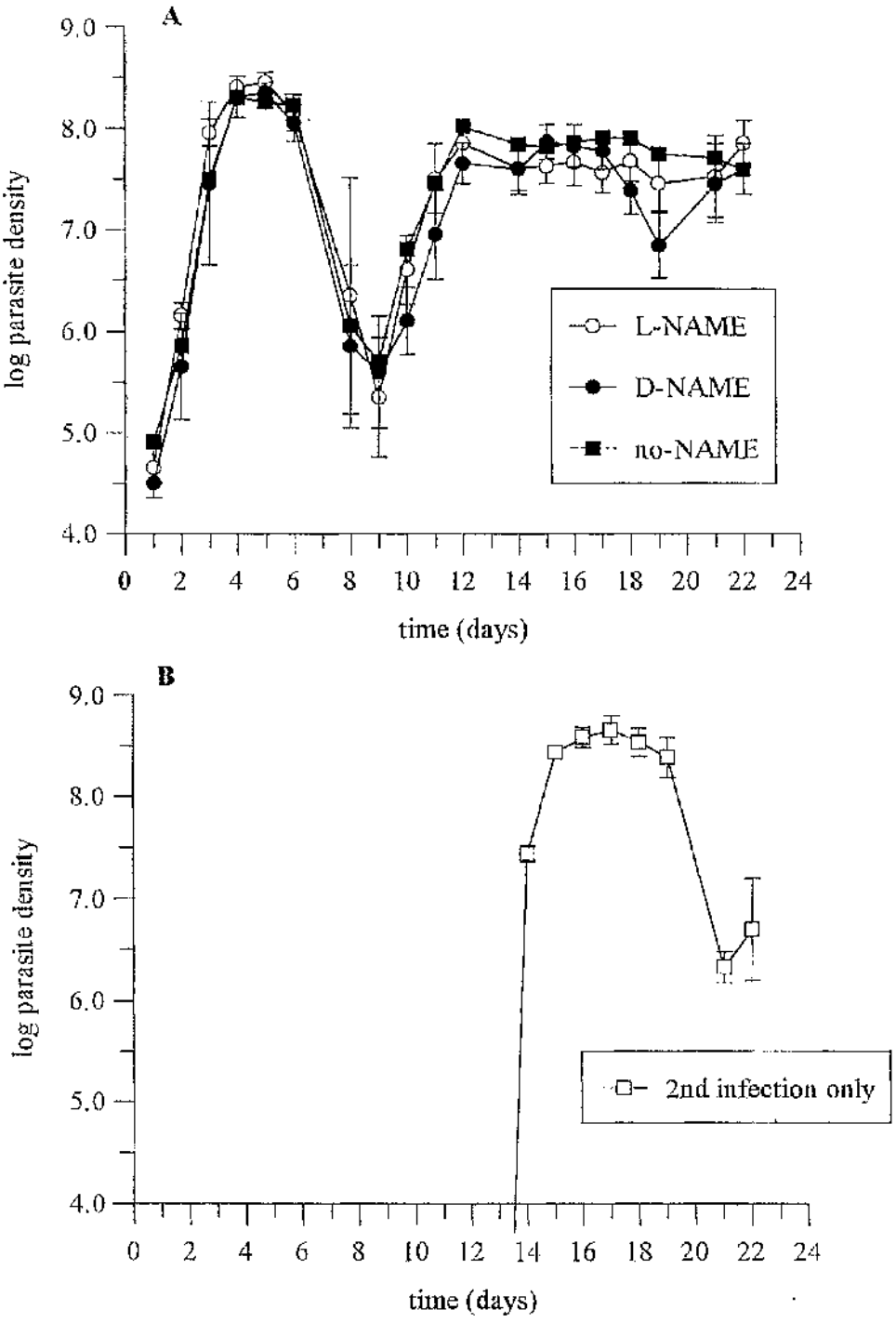


Fig 16. Effect of nitric oxide synthase inhibition upon parasite growth in mixed infections.
Parasitaemias in 4 groups of mice; after drug treatment with the NO synthase inhibitor, L-NAME, and in three control groups; D-NAME, no-NAME (Fig16A) and second infection (GUTat 7.2) only (Fig 16B). Drug treatments were administered daily from days 9 to 21. All groups shown in Fig 16A were given a secondary infection of GUTat 7.2 on day 12. Data points denote geometric mean +/- 2SE, n = 6

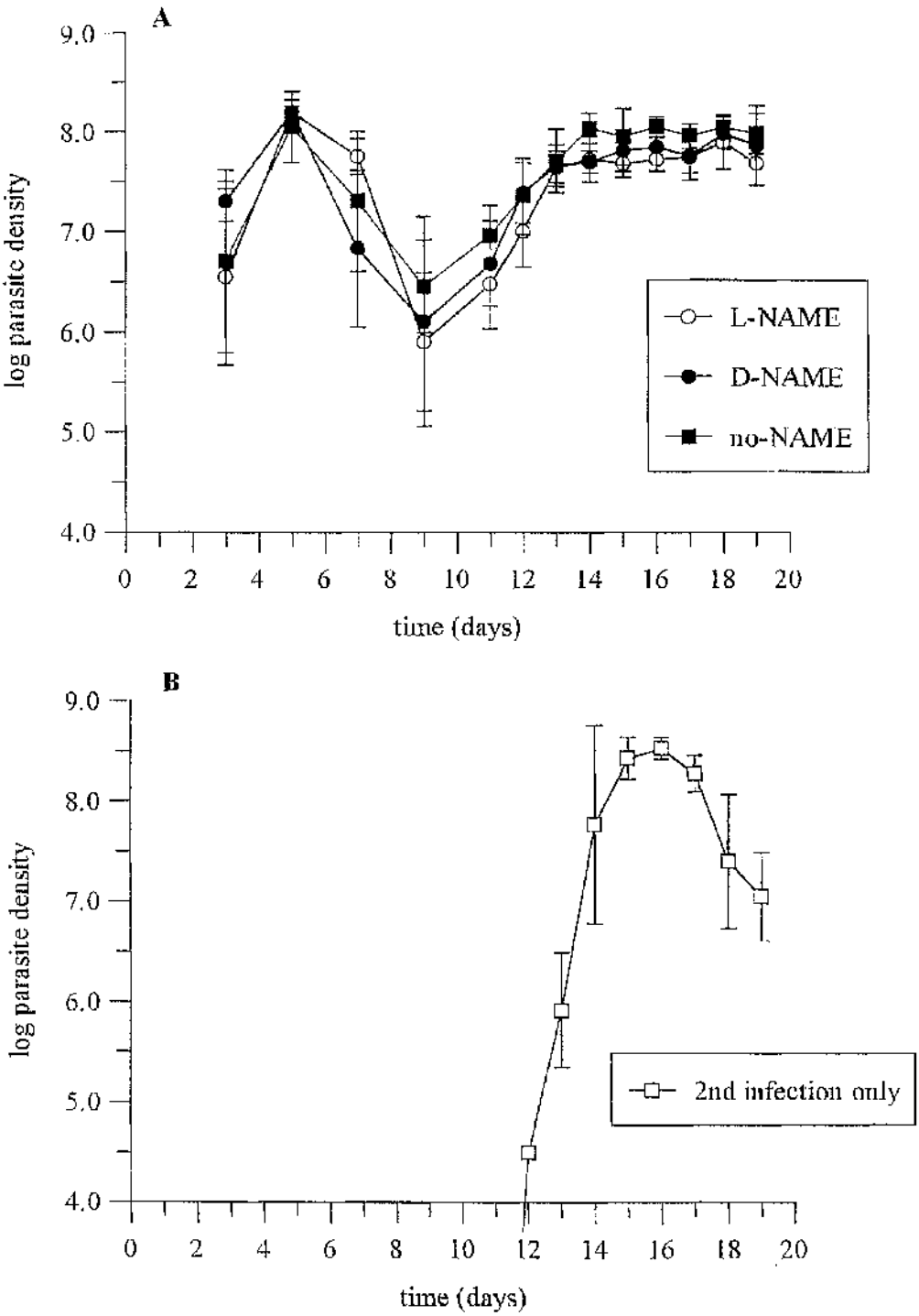


Fig 17. Effect of nitric oxide synthase inhibition upon parasite growth in mixed infections.
Parasitaemias in 4 groups of mice; after drug treatment with the NO synthase inhibitor, L-NAME, and in three control groups; D-NAME, no-NAME (Fig17A) and second infection (GUTat 7.2) only (Fig 17B) Drug treatments were administered daily from days 9 to 18. All groups shown in Fig 17A were given a secondary infection of GUTat7.2 on day 10. Data points denote geometric mean +/- 2SE, n = 6.

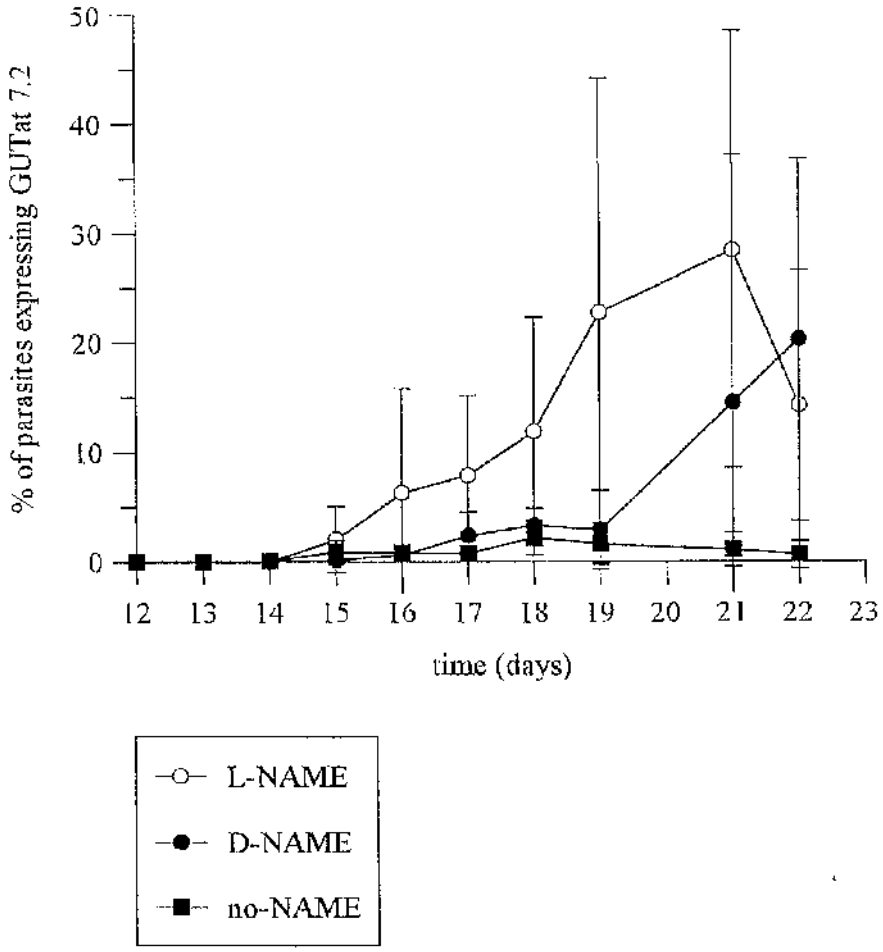


Fig 18. Effect of nitric oxide synthase inhibition upon parasite growth in a mixed infection
The proportion of the parasite population expressing GUTat 7.2. GUTat 7.2 trypanosomes were inoculated into mice already harboring EATRO 2340 infections on day 12 and differentiated from EATRO 2340 trypanosomes by immunofluoresence staining. Data points denote geometric mean +/- 2SE, n = 6.

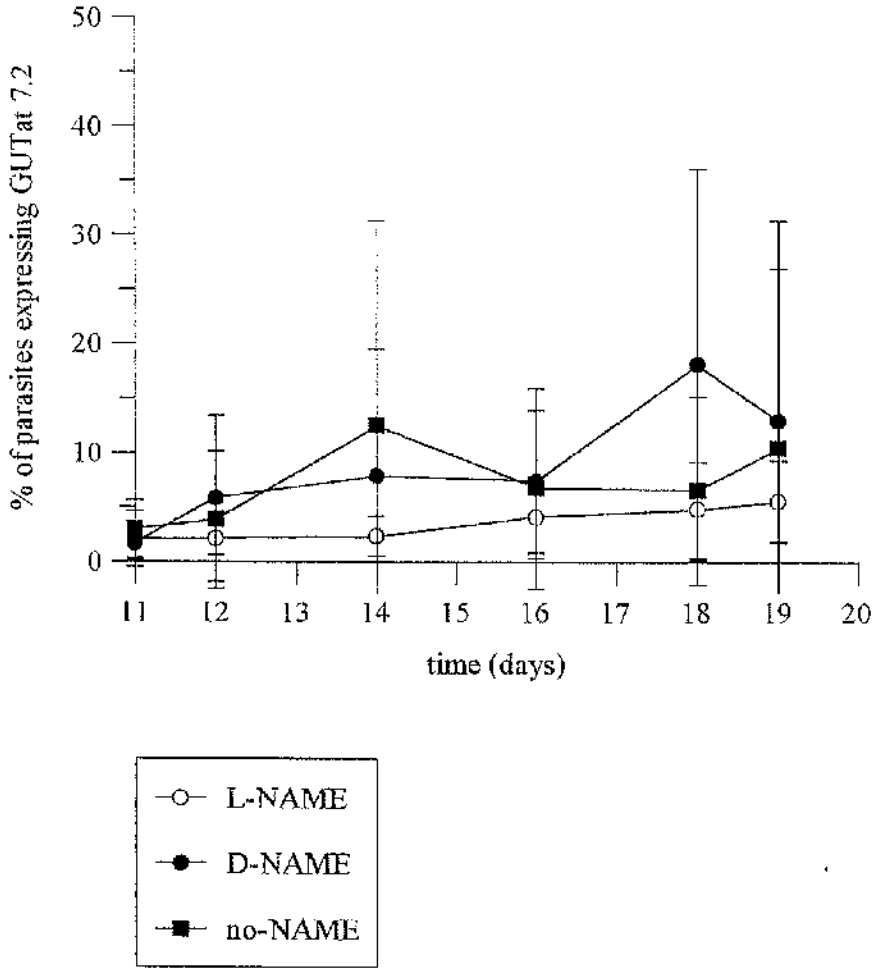


Fig 19. Effect of nitric oxide synthase inhibition upon parasite growth in a mixed infection.
The proportion of the parasite population expressing GUTat 7.2. GUTat 7.2 trypanosomes were inoculated into mice already harboring EATRO 2340 infections on day 10 and differentiated from EATRO 2340 trypanosomes by immunofluorescence staining. Data points denote geometric mean +/- 2SE, n = 6.

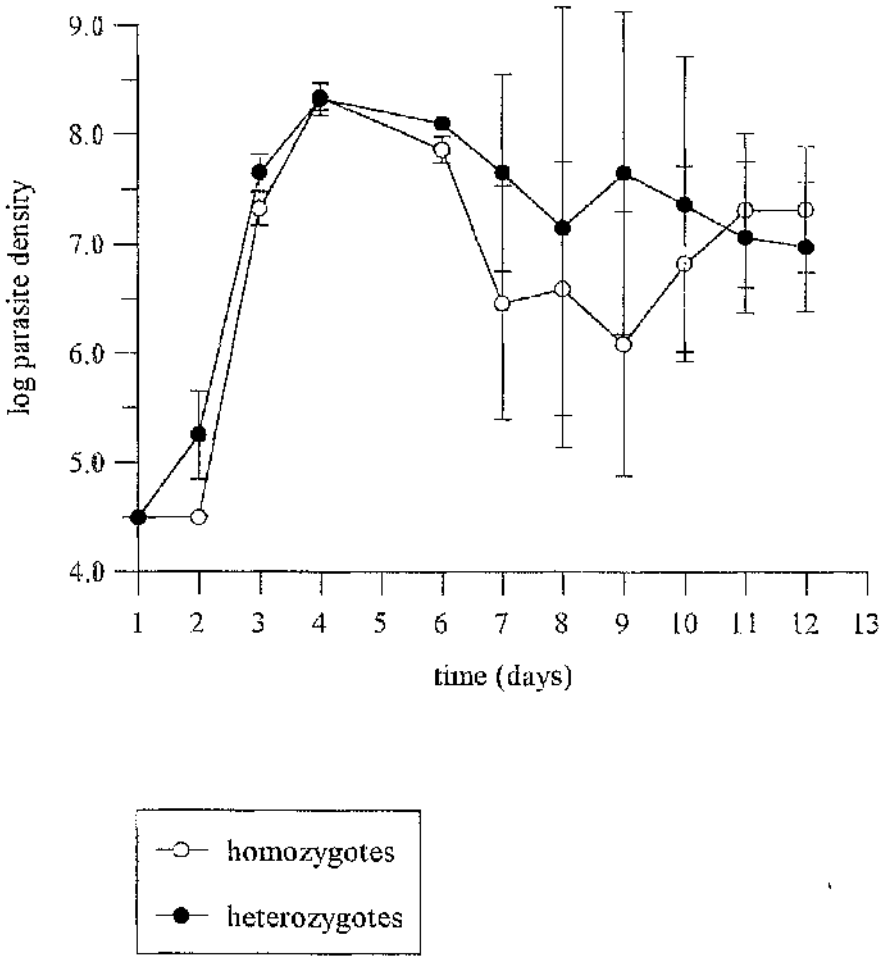


Fig 20. Effect of lack of inducible nitric oxide synthase activity upon trypanosome parasitaemias in mixed infections.
iNOS deficient and control mice received EATRO 2340 trypanosomes i.p. followed by GUTat 7.2 trypanosomes i.p. on day 7 of infection. Data points denote geometric mean +/- 2SE, n = 5 in the homozygotes and 4 in the control heterozygotes.

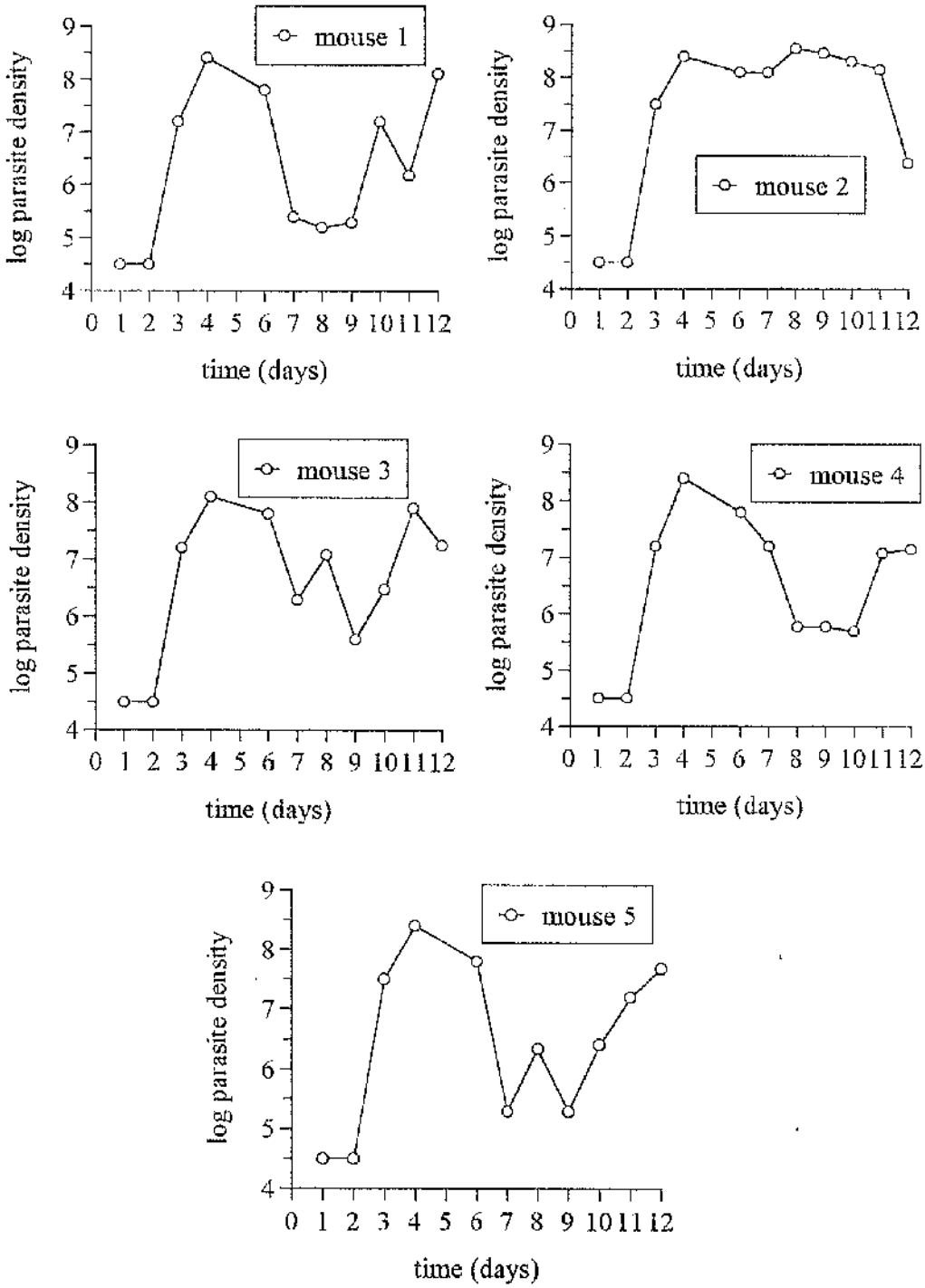


Fig 21. Effect of lack of inducible nitric oxide synthase activity upon trypanosome parasitaemias.
Parasitaemias in individual mice homozygous negative for the iNOS gene locus. Mice received EATRO 2340 trypanosomes i.p. followed by GUTat 7.2 trypanosomes i.p. on day 7 of infection.

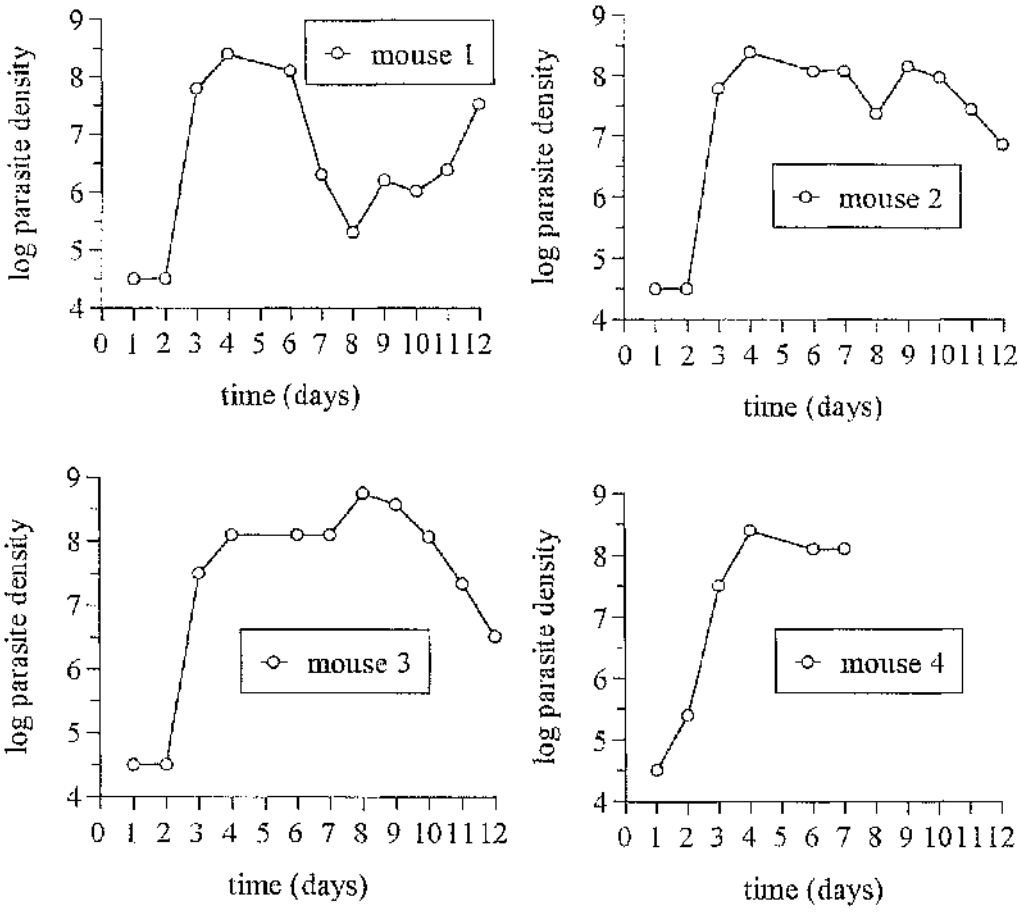


Fig 22. Effect of lack of inducible nitric oxide synthase activity upon trypanosome parasitaemias.
Parasitaemia in individual mice heterozygous for the iNOS gene locus. Mice received EATRO 2340 trypanosomes i.p. followed by GUTat 7.2 trypanosomes i.p. on day 7 of infection. Mouse 4 died on day 8 of infection.

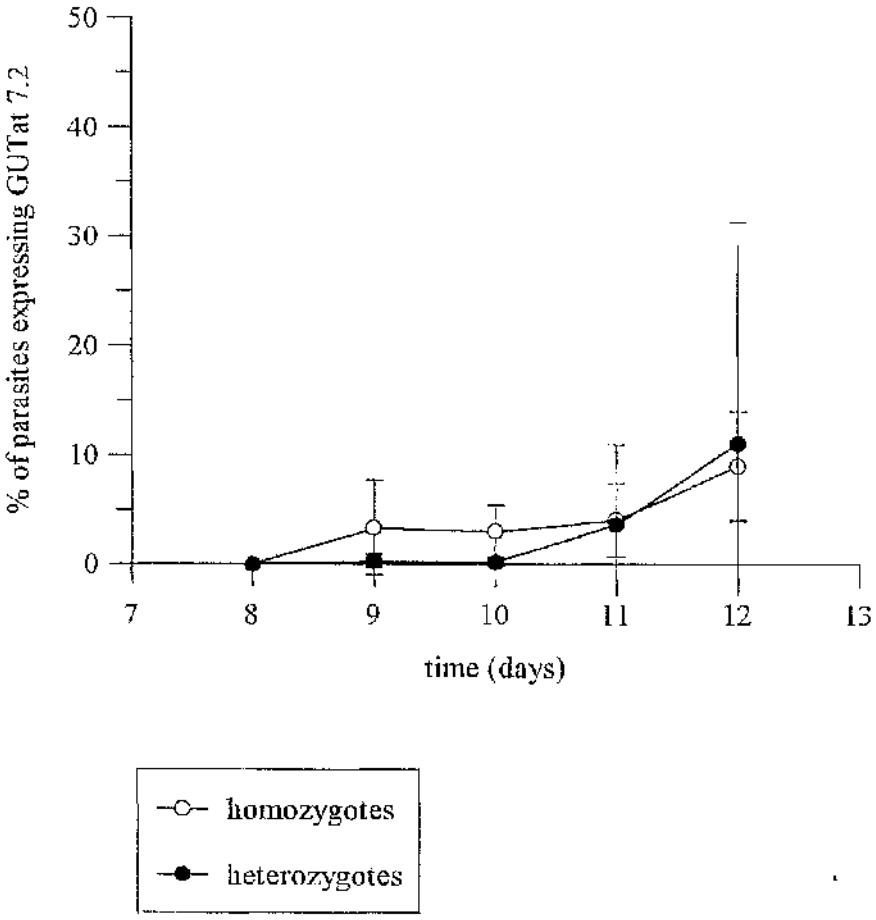


Fig 23. Effect of lack of inducible nitric oxide synthase activity upon growth of parasites in a secondary infection, expressing GUTat 7.2, in the context of a mixed infection.
The proportion of the total parasitaemia made up by GUTat 7.2 trypanosomes. GUTat 7.2 trypanosomes were differentiated from EATRO 2340 trypanosomes by immunofluorescence staining. Data points denote geometric mean +/- 2SE, n = 5 for homozygotes and 4 for heterozygotes.

genotype	plasma from day 4	plasma from day 8
-/-	< 1	24.2
-/-	25.6	85.2
-/-	52.6	83.1
-/-	< 1	60.1
-/-	24.9	23.5
mean	**21.0**	**55.2**
-/+	34.6	37.4
-/+	34.8	56.1
-/+	38.2	50.5
-/+	31.7	ND
mean	**34.8**	**48.0**

Table 1. Levels of nitrite plus nitrate detected in plasma obtained from individual mice homozygous and heterozygous for the iNOS gene locus infected with EATRO 2340 and GUTat 7.2 trypanosomes (nM). ND = not determined. Data for uninfected control plasma was not available in this experiment.

genotype	uninfected control plasma	plasma from day 5	plasma from day 11	plasma from day 16
-/-	57.9	45.1	over nM range	26.3
-/-	45.8	36.4	27.5	22.4
-/-	24.0	41.6	21.7	21.5
-/-	29.6	24.0	23.3	20.5
-/-	30.1	29.9	33.2	29.2
mean	**37.5**	**35.4**		**24.0**
+/-	43. 6	34.4	32.1	37.1
+/-	42.0	65.1	40.8	35.6
+/-	44.0	34.6	32.3	39.3
+/-	32.1	37.5	34.0	34.8
mean	**40.4**	**42.9**	**34.8**	**36.7**

Table 2. Levels of nitrate plus nitrite detected in plasma obtained from individual mice homozygous and heterozygous for the iNOS locus infected with STIB 247 and STIB 247 phleo trypanosomes (nM). No mean value could be determined for homozygous mice on day 11 of infection.

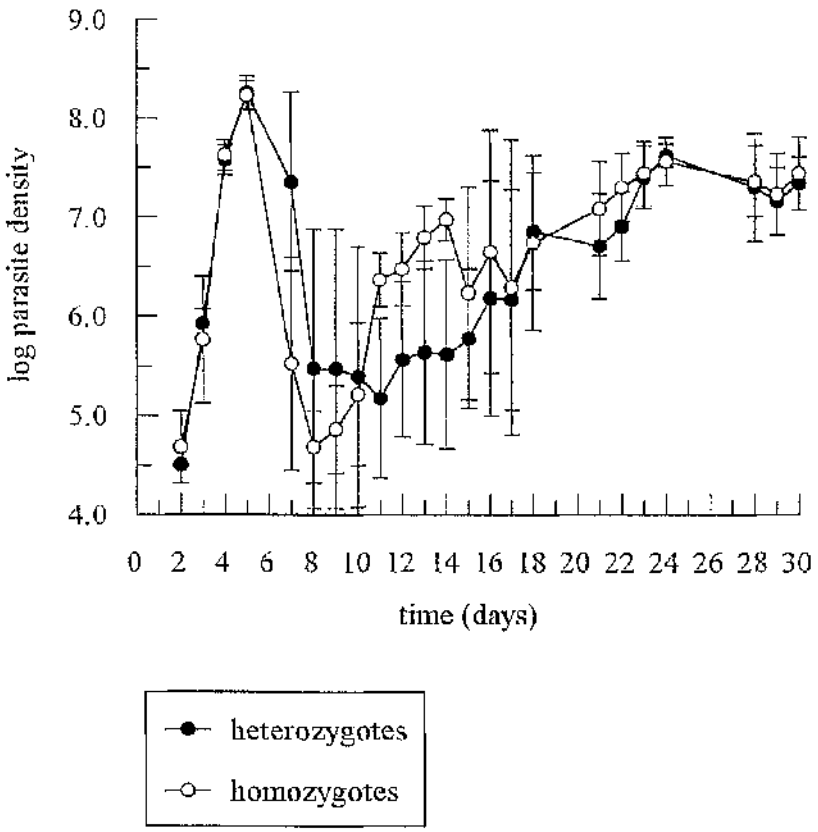


Fig 24. Effect of lack of inducible nitric oxide synthase activity upon trypanosome parasitaemias in mixed infections.
iNOS deficient and control mice received STIB 247 trypanosomes i.p. followed by STIB 247 phleo trypanosomes, i.p. on day 9 of infection. Data points denote geometric mean +/- 2SE, n = 5 in the homozygotes and 4 in the control heterozygotes.

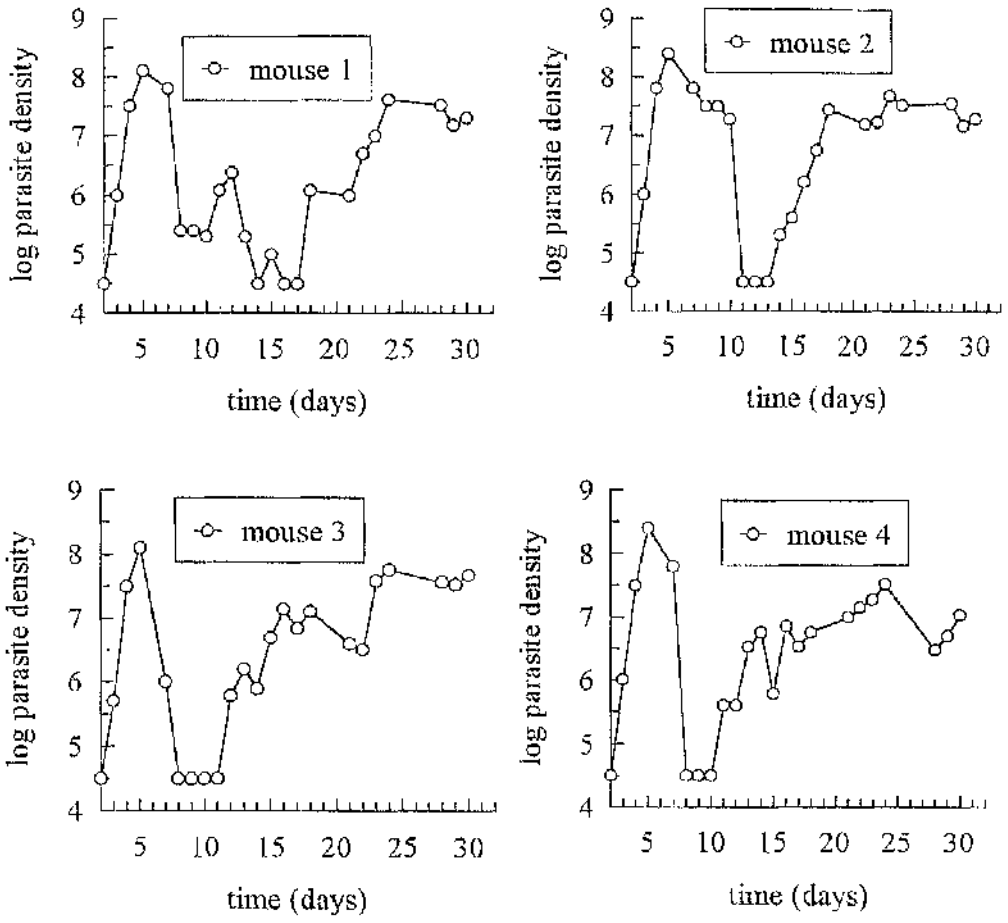


Fig 25. Effect of lack of inducible nitric oxide synthase activity upon trypanosome parasitaemias.
Parasitaemias in individual mice heterozygous for the iNOS gene locus. Mice received STIB 247 trypanosomes i.p. followed by STIB 247 phleo trypanosomes i.p. on day 9 of infection.

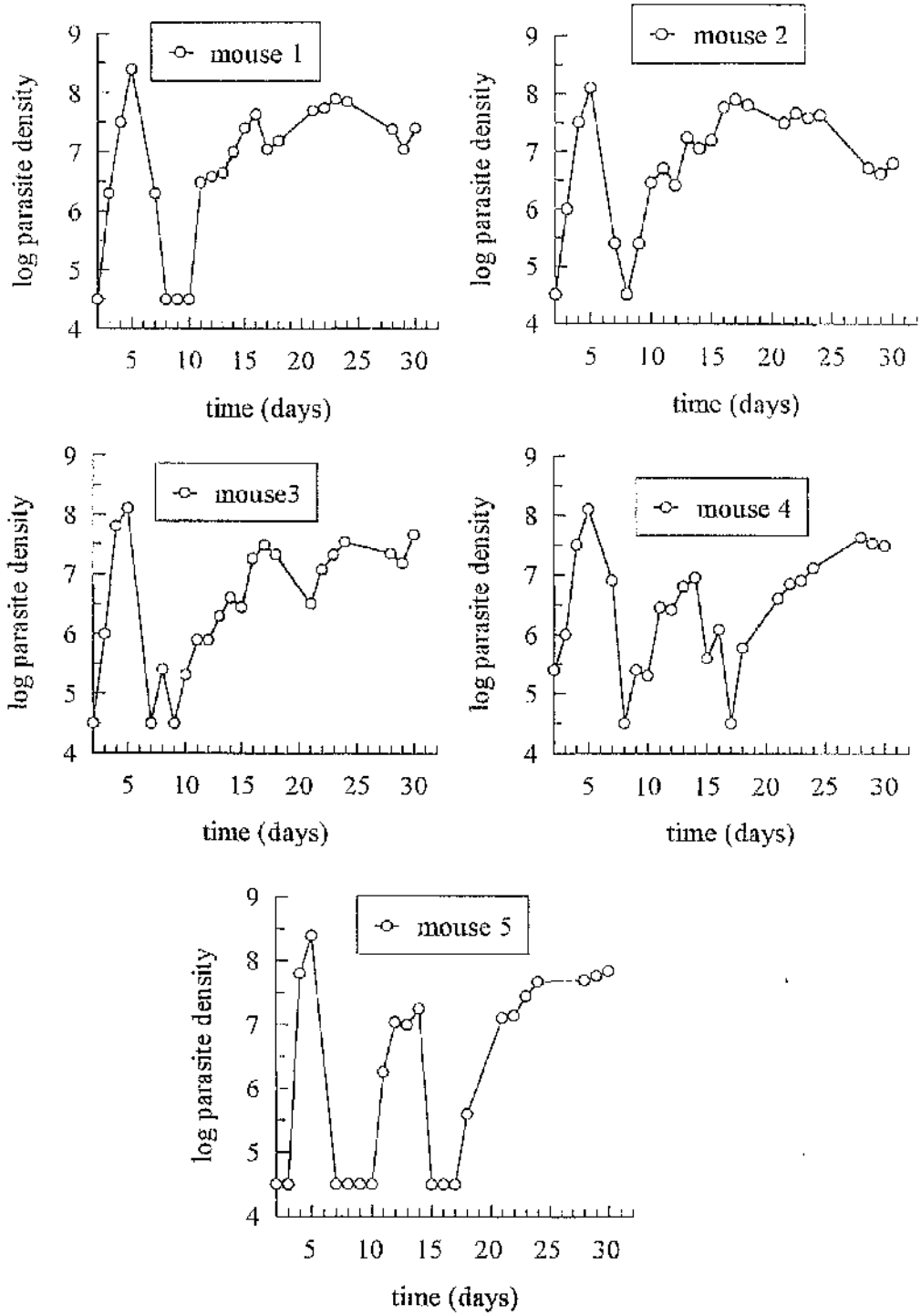


Fig 26. Effect of lack of inducible nitric oxide synthase activity upon trypanosome parasitaemias.
Parasitaemias in individual mice homozygous negative for the iNOS gene locus. Mice received STIB 247 trypanosomes i.p. followed by STIB 247 phleo trypanosomes i.p. on day 9 of infection.

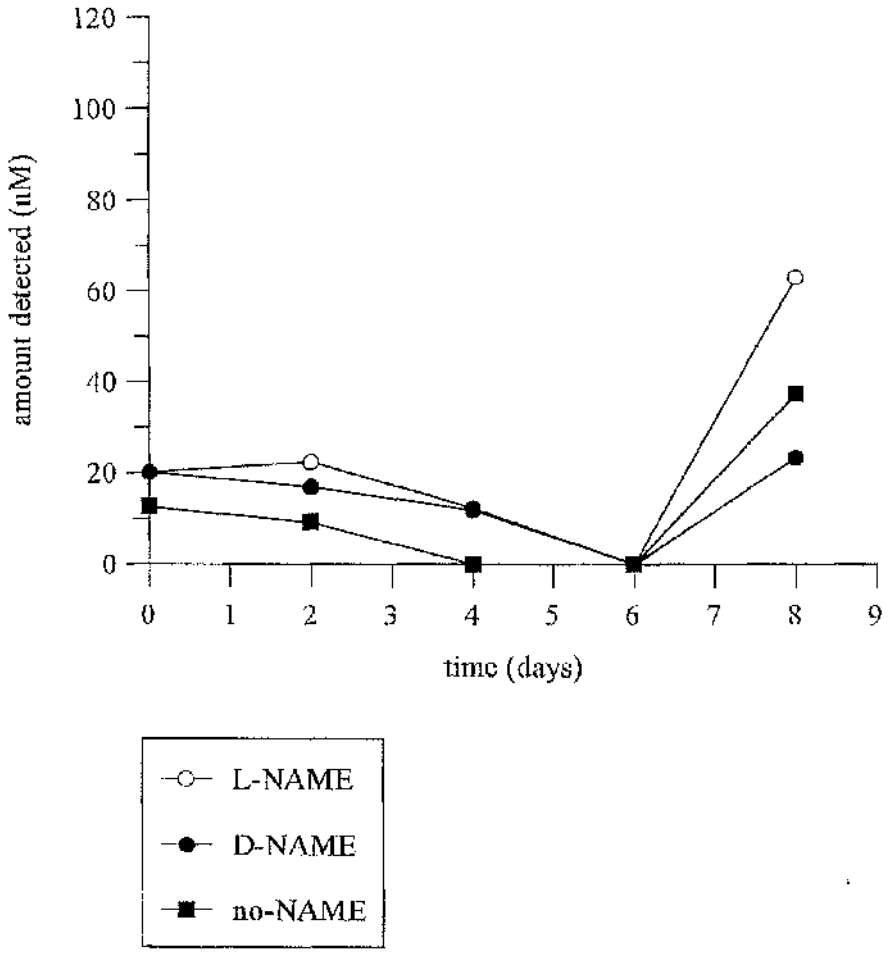


Fig 27. Effect of L-NAME upon nitrite produced in plasma during acute-phase infection with trypanosomes.
Amount of nitrite produced in the plasma of mice treated with the NO synthase inhibitor, L-NAME, and in control groups during infection. Nitrite content was assayed by the Griess assay.

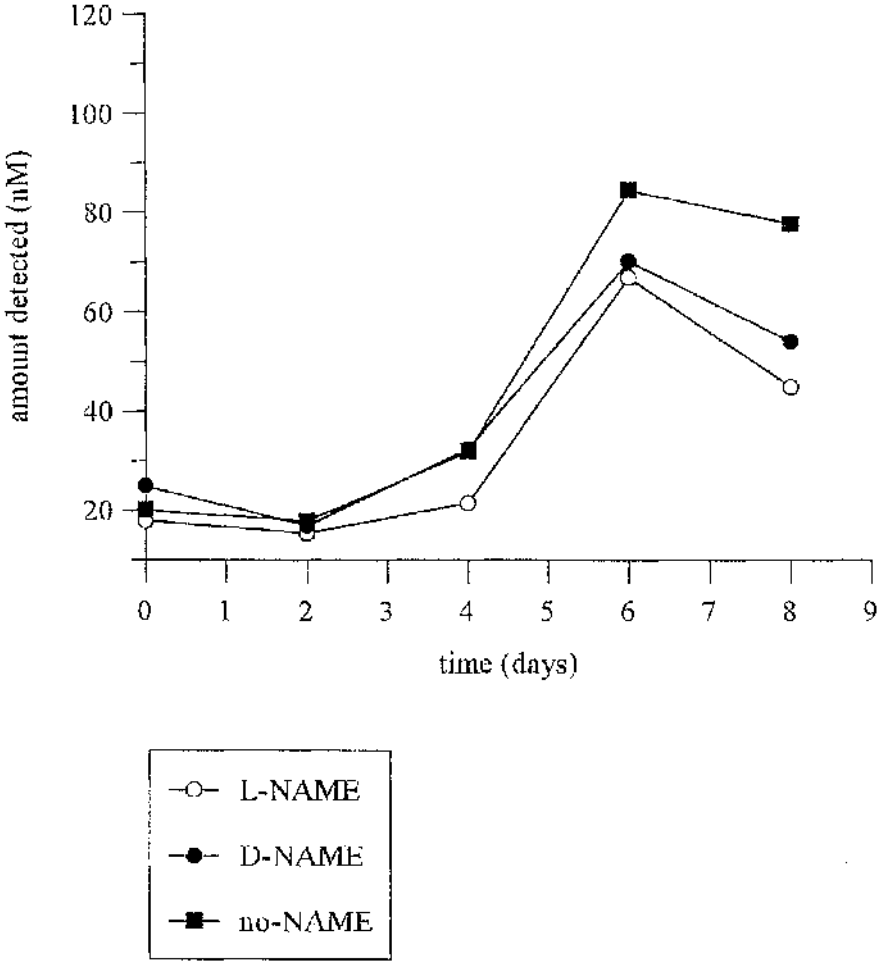


Fig 28. Effect of L-NAME upon nitrate produced in plasma during acute-phase infection with trypanosomes.

Amount of nitrate produced in the plasma of mice treated with the NO synthase inhibitor, L-NAME, and in control groups during infection. Nitrate was reduced to nitrite and assayed by the Griess assay.

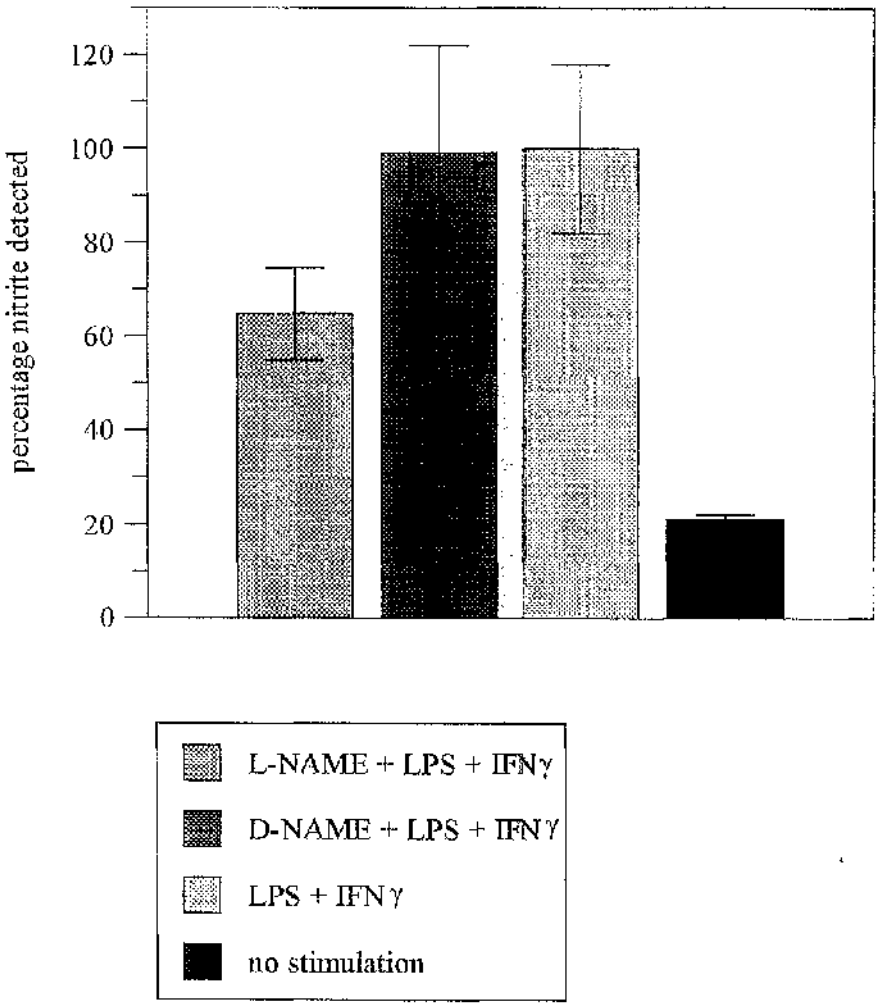


Fig 29. Amount of nitrite produced by stimulated peritoneal macrophages upon treatment with iNOS inhibitors *in vitro*.
Macrophages were cultured at 2 x 10^5 cells per well, stimulated with lipopolysaccharide and interferon gamma. The NO synthase inhibitor, L-NAME, and it's enantiomer, D-NAME, were each added at concentrations of 1 mM.

CHAPTER 4

IN VITRO CULTURE OF BLOODSTREAM FORM TRYPANOSOMES

4.1. Introduction

Culture of uninfective procyclic forms of *T.brucei* and *T.congolense* was achieved before that of bloodstream forms (reviewed by Hirumi *et al* 1980). *In vitro* culture of blood stage trypanosomes has been performed since the late 1960s. Originally, methods for bloodstream form culture had an absolute requirement for the presence of feeder cells. The use of feeder cells in the culture of trypanosomes was first introduced by Le Page who achieved short term growth of bloodstream forms with mouse L cells and mammalian tissue culture medium (Le Page 1967). It was several years until the continuous culture of infective monomorphic *T.brucei* was achieved (Hirumi *et al* 1977a, 1977b). Epithelial and fibroblast-like cell lines have been used (Hirumi *et al* 1980) in conjunction with RPMI 1640 medium supplemented with 20% heat inactivated Foetal Calf Serum (Hill 1978a, 1978b; Hirumi *et al* 1977a, 1977b; Hirumi *et al* 1980; Hecker and Brun 1982). Actively metabolising feeder cells of mammalian lineage had been shown to be essential to the growth of trypanosomes, supplemented RPMI medium alone did not support growth (Tanner 1980). Attempts to grow pleomorphic, tsetse-transmissible *T. brucei* stocks on feeder cell layers were largely unsuccessful until 1981 (Brun *et al* 1981).

In 1981 a culture system was devised using fibroblast-like cells derived from embryos of New Zealand White rabbits or *Microtus montanus* and HEPES -buffered Minimal Essential Medium, with Earle's salts and 15% inactivated rabbit serum. This allowed growth of mouse-infective bloodstream forms with an intact surface coat as demonstrated by electron microscopy. Cultures could be established with metacyclic forms from

Glossina m. morsitans harboring mature infections, bloodstream forms from mammalian hosts and from cryopreserved stabilates. Cultured trypanosomes could be cyclically transmitted through tsetse flies and the metacyclic forms returned to culture (Brun *et al* 1981).

Whilst the ability to culture bloodstream forms *in vitro* was a very significant technical development at the time, the use of feeder cell layers in trypanosomes culture was limiting in that cell yield was relatively low and the metabolism of trypanosomes could not easily be studied separately from that of feeder cells.

More recently, culture systems have been developed which allow growth of trypanosome bloodstream forms in the absence of feeder cells (Duszenko *et al* 1985, 1992; Hamm *et al* 1990; Hesse *et al* 1995; Hirumi and Hirumi 1989). Cysteine, incorporated into Minimal Essential Medium at levels of 1.5 to 3.0 mg/ml, was found to be critical to cell growth and to remove the absolute requirement for a feeder cell layer. The population doubling time in this culture system was significantly shorter that observed for the same clone in feeder layer supported cultures. At high levels however (> 20 mg/ml), cysteine proved toxic to trypanosomes and required addition of the reducing agents pyruvate or catalase (Duszenko *et al* 1985).

Other workers in the field developed culture systems allowing adaptation of bloodstream forms of *T. brucei*, *T. equiperdum*, *T. evansi*, *T. rhodesiense* and *T. gambiense* to culture and maintenance, for over 4 months; the trypanosomes grew in the absence of feeder cells. Trypanosomes were grown in Minimal Essential Medium (MEM) with

Earle's salts variously supplemented with L-glutamine, non-essential amino acids, HEPES, glucose, sodium bicarbonate, 2-mercaptoethanol, sodium pyruvate, hypoxanthine, thymidine and 10% heat inactivated serum from various sources. Trypanosomes grown in such semi-defined media exhibited characteristics of *in vivo* bloodstream forms; i.e. morphology, infectivity, antigenic variation and glucose-based metabolism (Baltz *et al* 1985). Variations of this culture medium have been developed by several groups over recent years, allowing maintenance and rapid growth of trypanosomes between the densities of 10^4 and 5 x 10^6 per ml (Duszenko *et al* 1992, Hirumi and Hirumi 1989, Duszenko *et al* 1985, Hesse *et al* 1995). Establishment of cultures always requires an initiation, an adaptation and a maintenance phase (Baltz *et al* 1985). Maintenance of cultures requires 37°C, 2-5% CO_2 (Baltz *et al* 1985, Hirumi and Hirumi 1989, Hesse *et al* 1995, Hamm *et al* 1990).

In the 1989 paper by Hirumi and Hirumi the use of bathocuproine sulfonate, to minimize the toxic effects of hydrogen peroxide produced by the autoxidation of L-cysteine, and a reduced level of serum protein was introduced. The role of cysteine in promoting trypanosomes growth has been found to be due to an absolute stereo-specific requirement by trypanosomes for cysteine as a growth factor and not merely as a reducing factor (Duszenko *et al* 1992, Hesse *et al* 1995). Cysteine is thought to be involved in protein synthesis and formation of glutathione and trypanothione (Fairlamb 1989).

Although trypanosomes have also been cultured on agarose matrix (Carruthers and Cross 1992, Vassella and Boshart 1996) fluid-phase axenic culture of trypanosomes offers the

most convenient system in which to conduct *in vitro* studies into many aspects of trypanosome biology; cell growth kinetics, metabolism, antitrypanosomal compounds and the interactions of trypanosomes with immune factors.

Axenic culture of trypanosomes was attempted in this project in order to establish a system for investigating the origins of the growth inhibition observed in chronic infections of trypanosomes. The culture system used was developed by Dr. J. M. Sternberg (Sternberg *et al* 1994). It was intended that once I had established the culturing methodology in this laboratory, the potential effects of different types of cells (B cells, T cells, macrophages) from host animals and secreted cell products (growth factors, cytokines) upon the growth of culture adapted trypanosome cell lines EATRO 2340 and STIB 247 would be investigated in cell culture.

4.2. Methods

4.2.1. Bloodstream form culture medium and batch testing of foetal calf serum

Trypanosomes of the lines STIB 247 and EATRO 2340 were maintained, as described by Sternberg *et al* (1994), in Minimal Essential Medium (MEM) with Earle's Salts (GIBCO).

The following stock solutions were made up in deionised distilled water, filter sterilised and stored in 5 ml aliquots at -20°C:

Substance	Stock concentration	Add to 100ml
HEPES (Sigma)	1 M pH 7.4	0.1 ml
Glucose (BDH)	25 %	0.5 ml
Hypoxanthine (Sigma)	100 mM	0.33 ml
Thymidine (Sigma)	16 mM	0.33 ml
Adenosine (Sigma)	7.5 mM	0.33 ml
2-Mercaptoethanol (Sigma)	50 mM	0.5 ml
L-Glutamine (Sigma)	200 mM	1 ml
Bathocuprione sulphamic acid (Sigma)	5 mM	1 ml
Sodium pyruvate (BDH)	100 mM	1 ml
L-cysteine (Sigma)	100 mM	1 ml
Threonine (Sigma)	40 mM	1 ml

The volumes indicated were added to 80 ml Minimal Essential Medium. 2 ml of 50x stock MEM Non Essential Amino Acids (GIBCO), 7.5 ml of heat inactivated foetal calf serum and 7.5 ml of heat inactivated horse serum (GIBCO) were added to bring the final concentrations of these components to:

Substance	Final concentration
HEPES	1 mM
Glucose	0.0125%
Hypoxanthine	1 mM
Thymidine	0.16 mM
Adenosine	0.075 mM

2-Mercaptoethanol	0.2 mM
L-Glutamine	2 mM
Bathocuprione sulphamic acid	0.05 mM
Sodium pyruvate	1 mM
L-cysteine	1 mM
Threonine	0.4 mM
MEM nonessential amino acids	1%
Heat Inactivated FCS	7.5%
Heat Inactivated Horse Serum	7.5%

This medium will hereafter be referred to as TM-1. Trypanosomes cultures were maintained at 37°C in 5% CO_2 in flasks (Greiner) with the caps loosened to allow gas exchange. Cultures were fed daily or when culture density reached 10^6 trypanosomes/ml by addition of fresh medium to bring culture density to approximately 5×10^4 trypanosomes/ml.

The repeatability of cultures of STIB 247 and EATRO 2340 was tested in TM-1 containing Foetal Calf Serum batch number 30Q5246S. STIB 247 was seeded at a density of 1.65×10^5/ml in both media whilst EATRO 2340 was seeded at a density of 1.22×10^5/ml. Cultures were monitored daily by gentle agitation of flask contents, aseptic removal of 0.1 ml of culture and counting using an Improved Neubauer Haemocytometer.

In a second experiment cell growth was compared in the medium described above containing different batches of foetal calf serum (GIBCO) originating from Australia, batch number 30q6951s, and New Zealand, 30g2646d. STIB 247 was seeded at a density of 8.83 x 10^4/ml in both types of media and also in media containing batch 30Q5246S heat inactivated foetal calf serum, hereafter termed A-FCS. EATRO 2340 was seeded at a density of 9 x 10^4/ml in all three types of media. Cultures were established in duplicate and maintained at 37°C and 5% CO_2 and were counted daily as described above.

4.2.2. Investigation into the effect of seeding density on growth of trypanosome cultures

5 ml cultures of STIB 247 and EATRO 2340 were seeded at the following densities per ml; 10^4, 5 x 10^4, 10^5, 5 x 10^5 and 10^6. Cultures were counted daily as previously described and growth curves compared.

4.2.3. Comparison of the growth rates of EATRO 2340 bloodstream forms in two different media

EATRO 2340 was raised from stabilate in BalbC mice. When trypanosomes were growing exponentially and consisted mainly of slender forms (at approximately 5 x10^7 trypanosomes per ml), blood was removed under halothane anesthesia into Carter's

Balanced Salt Solution containing 10 U/ml heparin. Trypanosomes were separated from the blood over a percoll gradient as follows;

8.55 g of sucrose (Fisher Scientific, UK) and 2.00 g of glucose (BDH) were dissolved in Percoll (Pharmacia) and filter sterilised. This was made up to 100 ml with sterile Percoll and the pH adjusted with sterile 1M HEPES to pH 7.4. 1 ml of blood was mixed with 4 ml of Percoll and 1.5 ml of Phosphate Buffered Saline and then centrifuged at 17,500 g in a swing out rotor at 4°C for 20 minutes.

Trypanosome growth in TM-1 (section 4.7.1.) was compared with that in the medium described by Scott (1995). This was made up as follows.

To 100 ml of Minimal Essential Medium was added,

Non-Essential Amino Acids 100x stock	1 ml
HEPES	600 mg
Glucose	100 mg
Sodium Bicarbonate (BDH)	220 mg
L-Glutamine	30 mg

This was made up to 110 ml with double deionised water, adjusted to 7.3 with 5 M NaOH and then filter sterilised. This is termed Incomplete medium.

5 mls of each of the following were made up:

Dithiothreitol (Sigma)	6.17 mg/ml
Sodium Pyruvate	22 mg/ml
Catalase (Sigma)	0.17 mg/ml
Adenosine	1.07 mg/ml

Guanosine (Sigma)	0.566 mg/ml
Thymidine	24.22 mg/ml
Hypoxanthine	2 mg/ml

Equal volumes of each solution were mixed together and filter sterilised. 73 ml of incomplete medium was added to 7 ml of the above solution and 20 ml of heat inactivated foetal calf serum to make a total of 100 ml complete blood stream culture medium, hereafter termed (BSCM). The two media are clearly very similar. The principal differences between them are that the medium of Scott (1995) contains dithiothreitol and catalase whereas the medium of Sternberg contains cysteine, 2-mercaptoethanol, bathocuprione sulphamic acid, threonine and heat inactivated horse serum.

Trypanosomes were seeded in sextuplet cultures at a density of 10^5 trypanosomes/ml and immediately counted using an Improved Neubauer Haemocytometer. Cultures were maintained at 37°C in 5% CO_2 in culture flasks and their trypanosomes counted daily.

4.3. Results

4.3.1. Repeatability of growth of EATRO 2340 and STIB 247 trypanosomes in culture.

The trypanosome lines STIB 247 and EATRO 2340 were grown in TM-1 containing Foetal Calf Serum batch 30Q5246S (A-FCS) (Fig 30).Growth of STIB 247 trypanosomes

was almost identical in both experiments. Similar overall growth rates and peak cell densities were observed in both experiments over 72 hours and no viable trypanosomes were counted in cultures in either medium after 72 hours.

Growth of EATRO 2340 was also broadly similar in both experiments. An increased rate of growth and slightly elevated peak cell density was however observed in experiment 2 over experiment 1. No stationary phase of growth was observed in the former experiment and cell numbers dropped more sharply than in the latter medium. A brief stationary growth phase was observed in experiment 1. No viable trypanosomes were counted in cultures of either experiment after 72 hours.

4.3.2. Comparison of effects of source of FCS on growth of trypanosomes

Trypanosomes growth was compared in TM-1 supplemented with A-FCS, Australian Foetal Calf Serum (Oz-FCS) or Foetal Calf Serum from New Zealand (NZ-FCS) (Fig 31). STIB 247 grew most rapidly in medium supplemented with heat inactivated A-FCS. In medium supplemented with serum from either Australia or New Zealand trypanosome populations were barely capable of maintaining number and growth was negligible or absent. No viable trypanosomes were observed in medium containing Australian FCS after 48 hours.

EATRO 2340 displayed the highest rate of growth in medium supplemented with heat inactivated A-FCS. Growth in media supplemented with serum from either Australia or New Zealand was much reduced with lowered peak cell densities as compared with A-

FCS supplemented medium. No viable trypanosomes were observed in the culture containing serum from New Zealand after 48 hours.

4.3.3. The effect of seeding density upon growth of trypanosomes in *in vitro* cultures.

EATRO 2340 trypanosome cultures seeded at different densities in the range 10^1 - 10^6 trypanosomes per ml all grew at similar rates, suggesting that seeding densities of cultures had negligible effect upon rate of exponential growth (Fig 32) Peak cell densities in cultures, although arrived at later in cultures of lower seeding density, were also similar. The only apparent difference between cultures was that when seeded at 10^4 trypanosomes per ml there was a lag phase for the first 24 hours before onset of exponential growth. No difference in rates of exponential growth were seen in cultures of STIB 247 trypanosomes seeded at 4×10^5, 10^5 and 5×10^5 cells per ml but the culture seeded at 10^6 cells per ml displayed a lowered rate of growth (Fig 33). The culture seeded at 10^4 cells per ml displayed an initial lag phase in growth after which the growth rate resembled that of cultures seeded at a higher density. Seeding density was seen to have little effect upon peak cell density although, as seen in EATRO 2340 cultures, this was achieved later in cultures seeded at lower starting densities.

4.3.4. Comparison of the growth rates of EATRO 2340 bloodstream form primary cultures in two different media

In this single experiment, negligible growth occurred in bloodstream forms of EATRO 2340 trypanosomes in both TM-1 and BSCM over the first 15 hours (Fig 34). Over the next 25 hours cell densities dropped by half in TM-1 but over the same time period cell densities in BSCM remained virtually unchanged.

4.4. Discussion

Axenic culture of bloodstream form trypanosomes requires mammalian serum in order to support cell growth. Rabbit serum has previously been described as the most effective (Baltz *et al* 1985). However due to financial pressures foetal calf serum (FCS) is more routinely used. Testing of different batches is vital to successful culture as variability among sources of FCS can render some batches unsuitable to supporting growth. The batch of FCS 30Q5246S supported levels of growth with a high degree of repeatability and so was selected for use until fresh batches had been tested for growth. FCS from Australia and New Zealand supported a lower level of growth when compared to heat inactivated FCS from Amanda Millar. This clearly demonstrated the preference exhibited by trypanosomes for growth in medium supplemented with heat inactivated over active FCS. It has been observed previously (Sternberg - personal communication) that culture adapted trypanosomes of the strains EATRO 2340 and STIB 247 grow best at cell densities between 5×10^4 and 5×10^6 cells per ml. At lower densities cells fail to grow. EATRO 2340 is more sensitive to this density-dependent effect than is STIB 247. Results

obtained in this study support this conclusion; growth of EATRO 2340 seeded at 10^4 cells per ml entered a stationary phase for a period of 24 hours before cell growth occurred. By comparison STIB 247 seeded at this level did grow, albeit at a lower rate than when seeded at a higher level. It has been concluded from growth curves obtained at a range of densities above 10^4 per ml that seeding density has little or no effect upon growth rates or peak cell densities.

Establishment of blood stream form trypanosomes in culture requires three phases: initiation (often in the presence of feeder cells), adaptation and maintenance. In the two media tested in this study, BSCM and TM-1, cell numbers were maintained over a period of 40 hours. Both media were deemed to maintain cell numbers to a similar extent.

A variety of media have been used throughout the history of culture of bloodstream forms of *Trypanosoma brucei*. Those used for culture with feeder cells have been simple in their composition. All have included mammalian serum. With the advent of axenic cultures the composition of media has become increasingly complicated. Cysteine has been found by several workers (Duszenko *et al* 1985, 1992) to be essential to trypanosome growth in the absence of feeder cells. Cysteine's essential role as a growth factor has been disputed by other workers and substitution with other reducing agents i.e. pyruvate, catalase, mercaptoethanol, thioglycerol, has been found to support growth (Hamm *et al* 1990). Cysteine is reduced very rapidly in culture to cystine which does not support the growth of trypanosomes, hence the presence of reducing agents in culture medium is required to maintain cysteine in it's oxidised state. Bathocuprione sulphamic acid is a copper chelating agent and reduces the oxidation of cysteine, whilst pyruvate neutralises the H_2O_2 produced upon the oxidation of cysteine (Duszenko *et al* 1985, 1992). The two media used in this study were similar in that they both contained amino acids, BSCM contained guanosine whereas TM-1 contained threonine but other than this amino acid content was the same. The two media contained different reducing agents but both contained cysteine.

Several difficulties inherent with this type of trypanosome culture were encountered in this study. One of these was a high level of contamination. Antibiotics have been used in some media (Baltz *et al* 1985) but when used in culture of pleomorphic form result in a reduced rate of growth (Turner - personal communication). The absence of antibiotics from cell culture makes maintenance of aseptic culture a far more difficult task. The most common form of contamination in this study was yeast. The majority of work in this field has been performed on monomorphic lines of culture adapted trypanosomes. The lines used by myself were pleomorphic. Culture of pleomorphic trypanosomes in the presence of feeder cells is more problematic than that of monomorphic lines (Brun *et al* 1981). Growth rates of monomorphic forms is faster (Duszenko *et al* 1992) and peak cell densities higher (Hesse *et al* 1995) than that demonstrated in this study (Figs 30, 32, 33). Adaptation of trypanosomes from bloodstream forms *in vivo* to axenic culture involving adaptation to culture conditions is likely to have an effect upon the biochemistry of the parasite. Primary cultures of trypanosomes isolated directly from blood were therefore postulated to provide a model for investigating the possible effects of immune effector cells upon growth of bloodstream trypanosomes which have not been altered by adaptation to axenic culture. The lack of growth of these trypanosomes in culture (Fig 34) has made any investigation into growth of trypanosomes using this system ineffective. It is reasonable to assume that the above difficulties with axenic cultures of pleomorphic trypanosomes are in part to blame for the lack of success experienced in this study. Axenic culture of trypanosomes was attempted in this study in order to facilitate the investigation of inhibition of growth of trypanosomes (Turner *et al* 1996). In the event of

culture of trypanosomes being successful it was intended to investigate the effects of immune effector cells and secreted cell products upon growth of trypanosomes cultures. Strategies to investigate these interactions would have included coculturing trypanosomes with T cells, B cells and macrophages; addition of cytokines or growth factors to cultures of trypanosomes and blockade of the action of these factors by addition of monoclonal antibodies against cytokines and growth factors to mixed cultures of trypanosomes and immune cells.

Extensive contamination occurring in axenic cultures was due to operator error and rendered axenic cell culture as a method facilitating investigation of trypanosome growth inhibition ineffective.

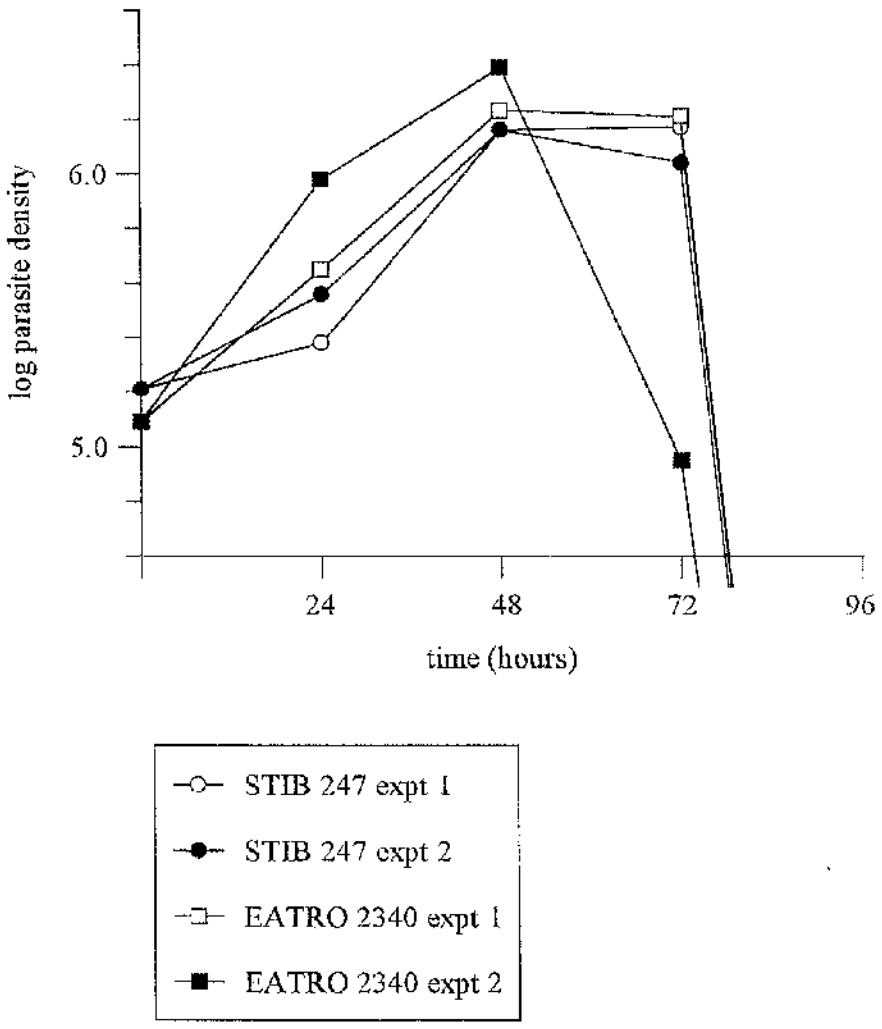
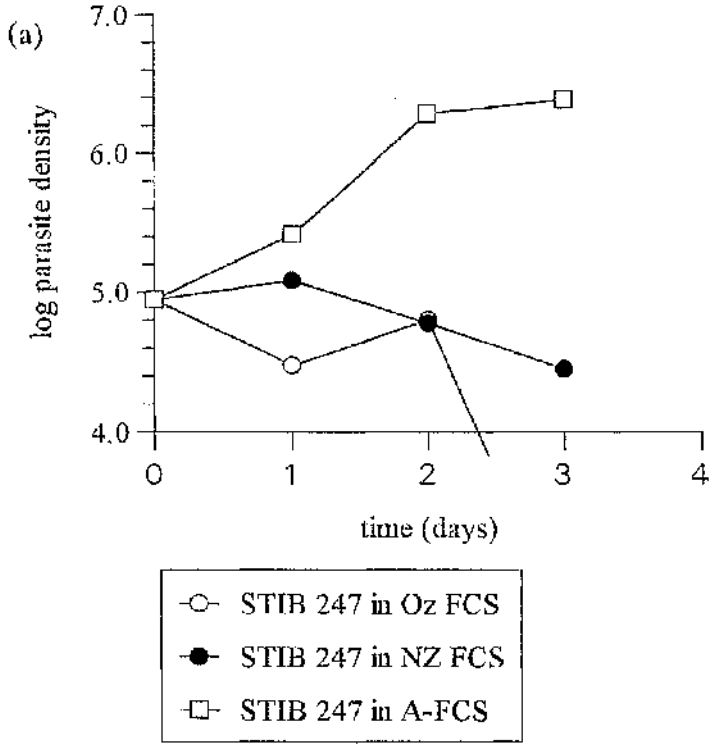


Fig 30. Growth of STIB 247 and EATRO 2340
Repeatability of growth of EATRO 2340 and STIB 247 in TM-1 containing A-FCS.



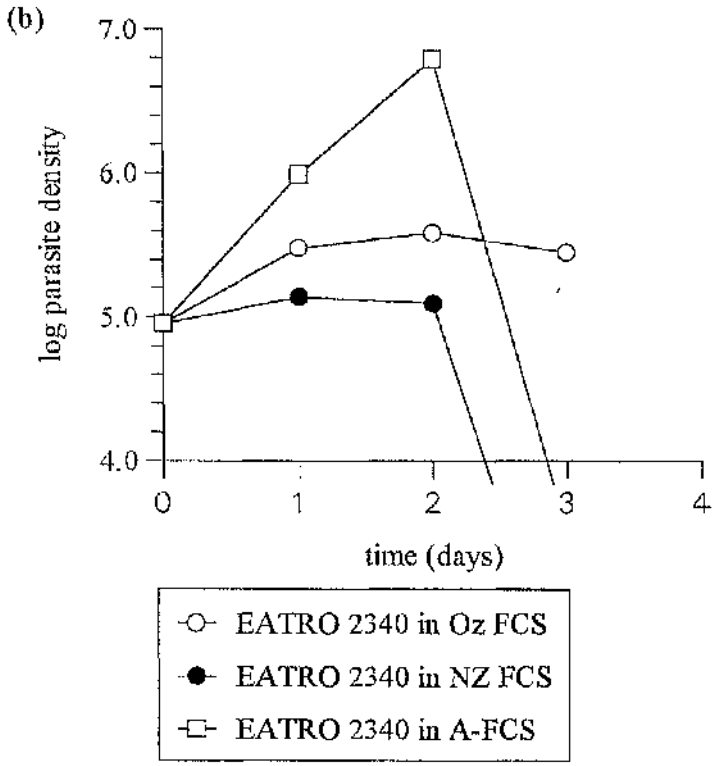


Fig 31. Comparison of growth of (a) STIB 247 and (b) EATRO 2340 in three Different Batches of Foetal Calf Serum
Geometric mean values are shown, n = 6.

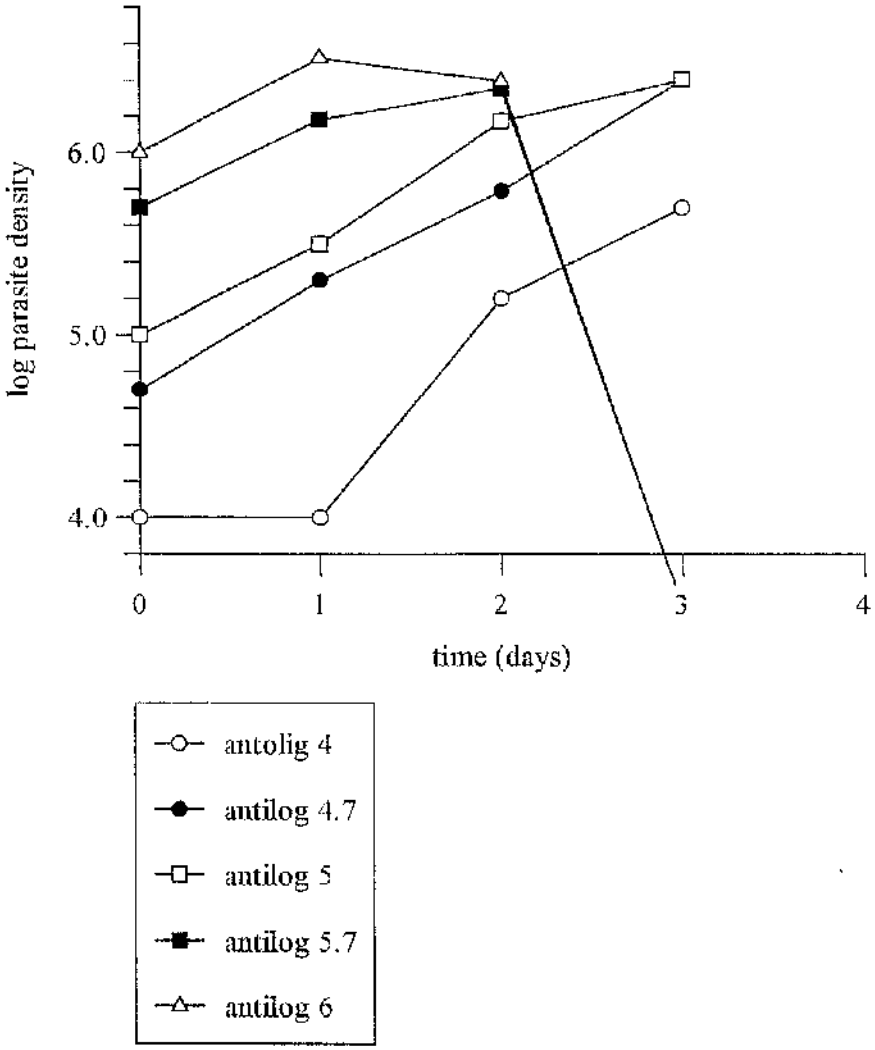


Fig 32. Growth of EATRO 2340 trypanosomes in cultures seeded at different densities

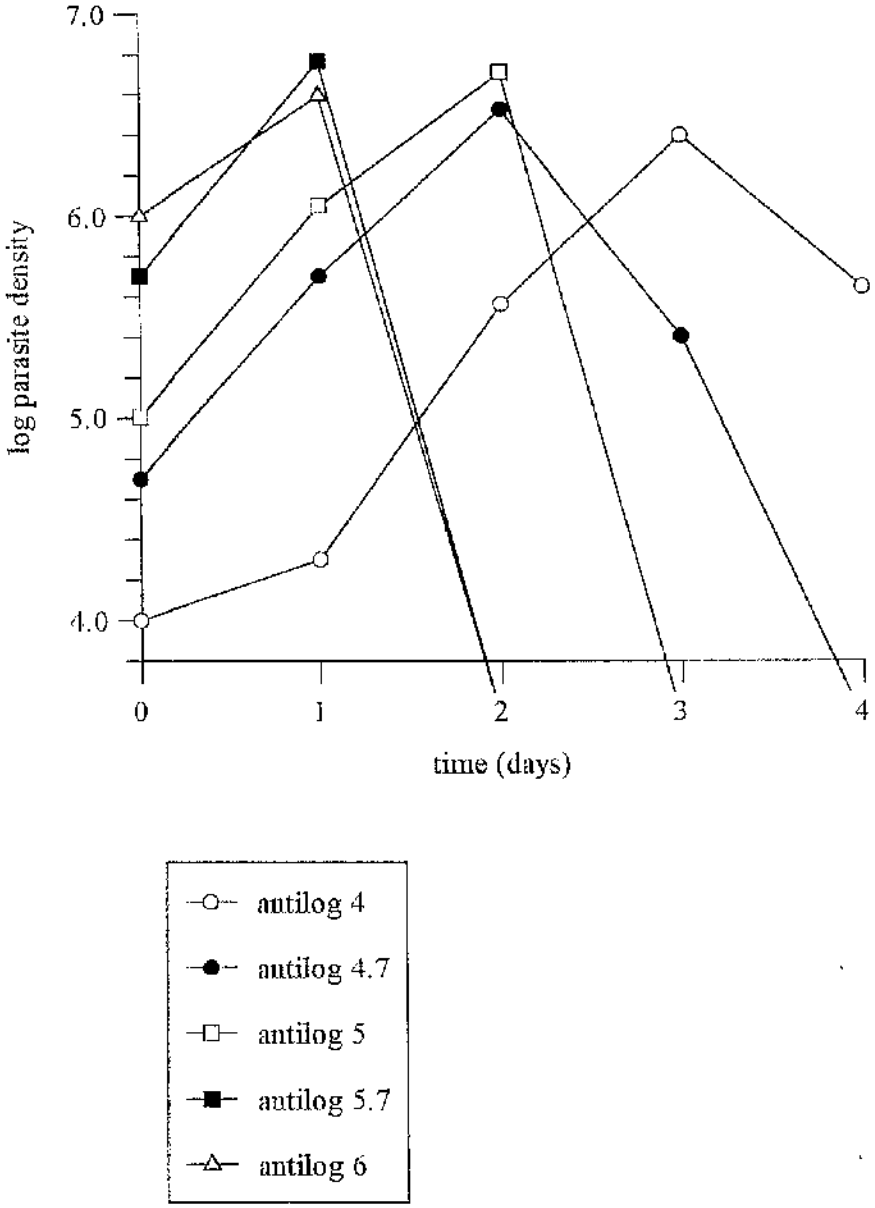


Fig 33. Growth of STIB 247 trypanosomes in cultures seeded at different densities

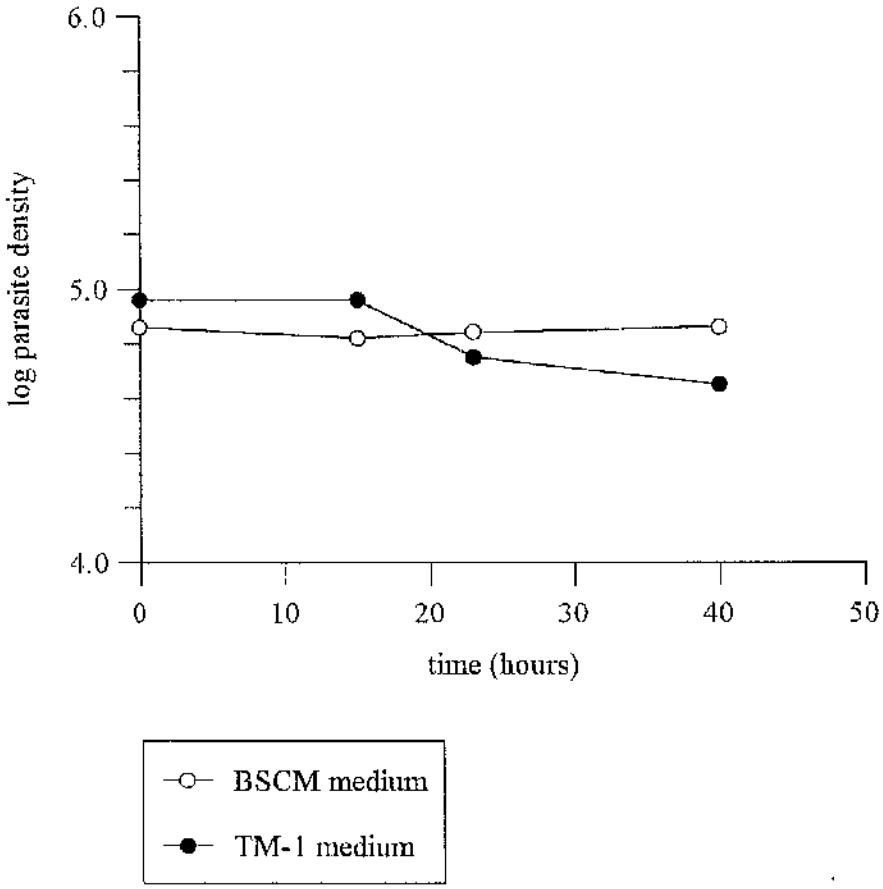


Fig 34. Comparison of the growth of primary cultures of EATRO 2340 bloodstream forms in two different media.
Geometric mean values are shown, n = 6.

CHAPTER 5

GENERAL DISCUSSION

It has been the purpose of this project to investigate regulation, and specifically inhibition, of the growth of trypanosomes during chronic infections with *T.brucei*. No factor responsible for inhibition of parasite growth was found to be transferable between mice with spleen cells suggesting that the putative inhibiting factor is of parasite origin (which I have argued is unlikely) or is of host origin but emanates from a radiation resistant type of cell. This latter argument arises because in transfer experiments, recipient mice were irradiated but the possible effects of any transferred resistant cells are likely to have been masked by the resident cells. Due to macrophages' radiation-resistant nature, and the dearth of literature concerning the antitrypanosomal actions of nitric oxide which is produced in substantial quantities in trypanosome-infected mice, macrophage-produced nitric oxide was investigated with regards to growth inhibition. No evidence was found in this work to support the hypothesis that nitric oxide was responsible for the regulation of parasite growth.

This is in direct contradiction with the indirect findings of other workers in the field. A cytostatic effect of NO upon *T.brucei* was demonstrated by Vincendeau and Daulouede (1991) and Vincendeau *et al* (1992) in *in vitro* culture conditions. Inhibition of inducible nitric oxide synthase activity has been shown by Sternberg *et al* (1994) to result in reduced parasitaemia. In this study, however, inhibition of iNOS activity was found not only to have little effect upon parasitaemia but to be incomplete using L-NAME drug treatment, the stratagem employed by previous workers. In addition, levels of nitric oxide metabolite detected in the blood of infected animals in this study were found to be significantly lower than those reported by other workers in the field (Sternberg - personal

communication). It is my opinion that the former discrepancy between this study and previous work is due to technical limitations with the experimental design in this project, particularly with respect to the efficacy of L-NAME as an iNOS inhibitor. Use of genetically manipulated mice to investigate the effect of lack of NO upon parasite growth also failed to indicate any effect of NO upon parasite growth. Even in these genetically manipulated mice there was incomplete removal of NO production, a possible cause of the failure to illustrate effect of lack of NO upon trypanosome growth.

In mixed infection experiments, growth inhibition of the parasites was observed to be density-dependent and to exist in two host species (mice and sheep) that have very different levels of parasitaemia (Turner *et al* 1996). In addition, transfer of spleen cells from infected to uninfected animals transferred a slight trend towards reduced growth although this could not be clarified, perhaps due to the postulated continued presence of competent resident macrophages in the irradiated recipient mice. These observations, taken together, are strongly suggestive of a host origin for the message signaling reduction in growth rate. A signal of parasite origin would not be demonstrated by transfer of splenocytes and would not be expected to generate grossly different levels of parasitaemias in mice and sheep. Transfer of a trend towards reduced rate of growth is suggestive that the putative factor may be a product of a cell of splenic origin.

Nitric oxide could not be demonstrated in this study to have a role in growth inhibition. This was in part due to failure to achieve a state *in vivo* of complete absence of nitric

oxide. Whilst a state of reduced nitric oxide led to a decrease in the degree of inhibition of growth in mixed trypanosome infections this was not readily reproducible and does not represent the situation which might occur in the total absence of elevated levels of NO. A similar situation exists in the case of NO synthase deficient mice; significant levels of NO were detected in mice homozygous for the iNOS gene deletion. The origin of the NO detected may be either incomplete deletion of iNOS gene activity, or compensatory upregulation of other isoforms of NO synthase activity.

In order to clarify the lack of a role for NO in the growth regulation of trypanosomes several improvements upon the experiments conducted in this study are suggested. In light of the ineffectiveness of L-NAME in inhibiting iNOS activity and the residual level of NO production in the iNOS knockout mice it would prove informative to investigate the growth of trypanosomes *in vivo* in an environment of total absence of elevated levels of NO. The range of disorders believed to involve a lack of, or deficiency, in NO suggest that such a state would not predispose to healthy animals and hence might give rise to questionable data. Strategies towards achieving improved depletion of NO levels in the host might include use of other inhibitors of iNOS. e.g. L-NMMA or other newer compounds still in the development stage such as amino-hydroxy-guanidine (Ruetten *et al* 1996). Further investigation of the role of macrophage activation in trypanosome growth and development of parasitaemia would be possible using antibodies to macrophage activation molecules both *in vivo* and *in vitro*, and by use of genetic mutants for such molecules.

The descriptions of the effects of NO upon the growth and survival of various protozoan blood parasites and the NO scavenging action of oxy-haemoglobin create a paradox. If oxy-haemoglobin is capable of scavenging the majority of free NO how does NO exert it attributed effects upon parasites? Three possible explanations exist. Firstly, it is possible that a low level of NO exists in a free state and that this low concentration is capable of carrying out NOs known actions. The second explanation is provided by the suggested allosteric control of haemoglobin's NO scavenging properties. Haemoglobin preferentially binds NO under oxygenated conditions. Upon deoxygenation, the cysteine group responsible for NO binding undergoes conformational change, placing it in a conformation and position whereby NO can be donated to the endothelial surfaces. Indeed NO release is found to be greater under conditions of deoxygenation. Specifically the s-nitrosyl-haemoglobin content of arterial blood was found to be 10 times greater than that of venous blood (Jia *et al* 1996). The third explanation is provided by the description of nitrosothiols by Jia *et al* (1996). Nitrosothiols are the products of nitrosylation of thiols in the blood to create a readily diffusable species which is resistant to scavenging by haemoglobin. The description of nitrosothiols in the blood with haemodynamic properties similar to NO but without the diffusional constraints exerted upon NO by oxy-haemoglobin (Jia *et al* 1996) raises interesting questions as to the properties of nitrosothiols in the context of parasite growth. If nitrosothiols have similar haemodynamic effects to NO might they not also duplicate the effects of NO upon trypanosomes so widely reported? It would be extremely interesting to establish the presence or absence of

cytostatic effects of nitrosothiols upon trypanosomes *in vitro* and *in vivo*. Addition of nitrosothiol inhibitors to infected animals would illustrate the effects of this species on trypanosome growth kinetics and would also allow investigation into immunosuppression in the absence of nitrosothiols. Culture of T cells and macrophages in the presence of exogenously added nitrosothiols would illustrate the involvement of nitrosothiols in the T cell suppressive activities currently attributed to NO.

Other products of activated macrophages include reactive oxygen species, prostaglandins and cytokines and these must all be considered possible candidates for inhibiting parasite growth. Cytokines produced by macrophages attract and activate neutrophils, T cells and NK cells and acts as growth factors for fibroblasts, endothelial cells and B cells (Abbas *et al* 1994). It is as yet unknown what, if any, effects these species may have on parasite growth in the vertebrate host or what interactions may exist between the products of macrophages and other factors known to have an effect upon parasite growth. In view of the protective effects of murine TNFα during the initial phase of infection (Magez *et al* 1993) and the trypanosome growth enhancing effects of EGF (Sternberg and McGuigan 1994) and transferrin (Coppens *et al* 1987), the interactions of reactive oxygen species and nitric oxide with transferrin, EGF and TNF provide an interesting area for investigation.

Trypanosomes cause a dose- and time-dependent increase in the production of PGE_2 and PGD by cultured murine fibroblasts (Alafiatayo *et al* 1994). In addition, raised levels of $PGF_{2\alpha}$ have been demonstrated in the plasma of *T. congolense* infected goats and PGD_2

levels are raised in the cerebrospinal fluid of chronically-infected humans (Alafiatayo *et al* 1994). Macrophage-produced prostagladins have been implicated in the T cell suppression characteristic of infection (Schleifer and Mansfield 1993). It remains to be seen what effect prostaglandins have upon the growth of trypanosomes. An indirect effect promoting growth of trypanosomes through T cell mediated immunosuppression is likely but a direct effect on parasite growth is distinctly possible. Another class of immune chemicals with unknown involvement in trypanosomiasis are the acute phase proteins. I am unaware of any data to date that have been published regarding the effects of these chemicals during infection.

In 1983 a study on the survival times of mice congenitally deficient for different immune cell types contained evidence that NK cells may be detrimental to survival of infection (Jones and Hancock 1983). In spite of this result no further work has been published with regard to the role that NK cells may play in the immune response to, and the regulation of growth of, trypanosomes that I am aware of. In light of this, co-culture of trypanosomes and NK cells in order to establish an effect upon trypanosome growth or survival followed by attempts to identify a factor or interaction would prove to be of interest. Investigation of the interactions of NK cells with other types of immune cells would provide insights into the mechanisms whereby NK cells reduce the survival of infected animals.
The approaches outlined above all assume that a molecule responsible for growth inhibition can be defined if the cell type producing the molecule can be identified, which, given the extensive knowledge base for the candidate cell types is reasonable. An

alternative approach would involve identifying the ligand molecule by inference from identification of it's receptor in the parasite. This approach assumes that there is a specific receptor for the inhibitory signal and that methodology for identifying it can be developed.

In an investigation into trypanosome growth by Turner *et al* (1996), superimposing one strain of trypanosome upon a pre-existing infection of an antigenically distinct strain resulted in inhibition of growth of the secondary infection. Inhibition of replication was not confined to the secondary strain but was exerted upon the entire trypanosome population. In an extension of that study (Milligan 1996) mutagenesis of GUTat 7.2 trypanosomes by incubation with ethylmethanesulphonate (EMS) *in vitro* gave rise to growth mutants. When used to establish acute-phase infections of mice these growth mutants displayed a lag phase in growth rate from which they recovered after day 3 post infection and then gave rise to a relapsing infection with parasitaemias similar to those of controls. When the growth mutants were employed as the secondary strain in a mixed infection however they were able to overcome the growth inhibition. These findings indicate that the tendency to display growth inhibition, or the response of a trypanosome population to a growth inhibitory signal, is at least in part dependent upon the characteristics of that trypanosome strain. In other words, whilst there may be a host-derived factor responsible for growth inhibition, the response of the trypanosome to host derived factors is also an important determinant of the parasite's growth rate. Mutations that affect the response of an organism to host-derived growth factors or inhibitors may result in changes in receptors to such factors on the parasite's surface.

Equally the mutation may reveal itself as a lesion in the signal transduction pathway downstream of the receptor. Alterations that might give rise to resistance to growth inhibition could include; a constitutively active receptor to a growth factor, amplification inducing modifications downstream of that receptor, deletion of or functionally subactive receptors for a growth inhibitory factor or a lesion in the signal transduction pathway downstream of that receptor.

EMS generates single point mutations, affecting one gene or allele. It would be of great interest to identify which gene is affected. In order to do this Differential Display might be employed to examine the two different RNAs produced by a growth mutant and the wild type. In this technique the RNA from the two strains is isolated. Various parts of the RNA is labeled at random and removed. These pieces of RNA are then run on an agarose gel. Bands present in the RNA of one strain but not the other are then removed and sequenced. A major potential problem with this technique is the large number of artifacts generated in the procedure, all of which are likely to give rise to bands which will be present in the RNA of one strain and not the other. Hence every band of interest must be double checked to ascertain that it is not due to an artifact. As the labeling of RNA is random, the gene which has been altered may not be labeled first time and hence this procedure must be repeated until a legitimate difference is isolated between the two strains.

It was the intention of this project to develop an *in vitro* cultivation system for growth of trypanosomes in order to ascertain if spleen cells or serum from infected animals were

responsible for the growth inhibition of trypanosomes seen in chronic infections. This was not successful. Had axenic culture of trypanosome been possible the following strategies would have been employed to investigate growth inhibition. A soluble factor present in the blood of infected mice with an effect upon growth would have been investigated by addition of serum or plasma from infected mice to trypanosomes cultures. Cocultures of spleen cells and trypanosomes would have been used to demonstrate a factor either secreted by spleen cells or present as a molecule on the surface of cells whilst a factor secreted by these cells might be investigated using the supernatants of spleen cell cultures. Subsequent to a factor being indicated by any of the previous tactics an attempt would have been made to characterize and identify it. Initially investigation into the nature of such a factor would focus on size, thermostability and resistance to protease and peptidase activity. In view of work by other researchers regarding the involvement of macrophage produced NO it would prove extremely interesting to employ the approaches described above to investigate the products of this cell type. Axenic culture would also have enabled investigation of the effects upon trypanosome growth of the prostaglandins, cytokines, nitrosothiols and reactive oxygen species previously discussed.

References

Abbas AK, Lichtman AH, Pober JS. (1994), Cellular and Molecular Immunology, 2nd edition. (Editors; Abbas AK, Lichtman AH, and Pober JS), W. B. Saunders Company, Philadelphia, London, Toronto, Montreal, Sydney, Tokyo.

Alafiatayo RA, Cookson MR, Pentreath VW. (1994)Production of prostaglandins D2 and E2 by mouse fibroblasts and astrocytes in culture caused by *Trypanosoma brucei brucei* products and endotoxins. *Parasitology Research* **80**:223-9.

Albright JW, Stewart MJ, Latham PS, Albright JF. (1994)Antibody - facilitated macrophage killing of *Trypanosoma musculi* is an extracellular process as studied in several variations of an *in vitro* analytical system. *Journal of Leukocyte Biology* **56**:636-643.

Askonas BA. (1985)Macrophages as Mediators of Immunosuppression in Murine African Trypanosomiasis. *Current Topics in Microbiology and Immunology* **117**:119-127.

Askonas BA, Bancroft GJ, (1984)Interaction of African trypanosomes with the immune system. *Philosophical Transactions of the Royal Society of London* **307**:41-50.

Aslam N, (1992)Growth, differentiation and the regulation of *Trypanosoma brucei* infections. Unpublished thesis, University of Glasgow.

Bagasra O, Schell RF, Le Frock JL. (1981)Evidence for Depletion of Ia^+ Macrophages and Associated Immunosuppression in African Trypanosomiasis. *Infection and Immunity* **32**:188-193.

Bakhiet M, Olsson T, Edlund C, Hojeberg B, Holmberg K, Lorentzen J, Kristensson K. (1993)A *Trypanosoma brucei brucei*-Derived Factor that Triggers CD8+ lymphocytes to Interferon-gamma Secretion: Purification, Characterisation and Protective Effects In Vivo by Treatment with a Monoclonal Antibody against the Factor. *Scandinavian Journal of Immunology* **37**:165-78.

Bakhiet M, Olsson T, Van Der Meide P, Kristensson K. (1990)Depletion of $CD8^+$ T cells suppresses growth of *Trypanosoma brucei brucei* and interferon-gamma production in infected rats. *Clinical and Experimental Immunology* **81**:195-9.

Bales JD, Harrison SMJ, Mbwabi DL. (1989)Treatment of arsenical refractory Rhodesiense sleeping sickness in Kenya. *Annals Of Tropical Medicine And Parasitology* **83**:111-4.

Baltz T, Baltz D, Giroud C, Crockett J. (1985)Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodesiense* and *T. gambiense*. *Embo Journal* **4**:1273-7.

Barrett J. (1997)Control Strategies for African Trypanosomes: Their Sustainability and Effectiveness. In: Trypanosomiasis and Leishmaniasis: Biology and Control (Editors: Hide G, Mottram JC, Coombs GH, Holmes PH), p347-59, CAB International

Barry JD. (1997)The Relative Significance of Mechanisms of Antigenic Variation in African Trypanosomes. *Parasitology Today* **13**:212-218.

Barry JD, Emery DL. (1984)Parasite development and host responses during the establishment of *Trypanosoma brucei* infection transmitted by the tsetse fly. *Parasitology* **88**:67-84.

Barry JD, Turner CMR. (1991)The dynamics of antigenic variation and growth of african trypanosomes. *Parasitology Today* **7**:207-11.

Barry JD, Vickerman K. (1979)*Trypanosoma brucei*: Loss of Variable Antigens during Transformation from Bloodstream to Procyclic Forms *in vitro*. *Experimental Parasitology* **48**:313-24.

Bhattacharya S, Manna PP, Bandyopadhyay S. (1993)Interferon-gamma is directly Cytostatic to the Extracellular Form (Promastigote) of *Leishmania donovani*. *Scandinavian Journal of Immunology* **37**:680-3.

Black SJ, Murray M, Shapiro SZ, Kaminsky R, Borowy NK, Musanga R, Otreno-Omundi F. (1989)Analysis of *Propionibacterium acnes*-induced non-specific immunity to *Trypanosoma brucei* in mice. *Parasite Immunology* **11**:371-383.

Black SJ, Vandeweerd V. (1989)Serum lipoproteins are required for multiplication of *Trypanosoma brucei brucei* under axenic culture conditions. *Molecular And Biochemical Parasitology* **37**:65-72.

Borowy NK, Sternberg J, Schreiber D, Nonnengasser C, Overath P. (1990)Suppressive macrophages occurring in murine Trypanosoma brucei infection inhibit T-cell responses *In vivo* and *In vitro*. *Parasite Immunology* **12**:233-46.

Brannan LR, Turner CMR, Phillips RS. (1994)Malaria parasites undergo antigenic variation at high-rates *in vivo*. *Proceedings Of The Royal Society Of London Series B-Biological Sciences* **256**:71-5.

Brun R, Schoenbechler MJ, Schell KL. (1981)*In Vitro* Cultivation of Bloodstream Forms of *Trypanosoma brucei*, *T. rhodesiense* and *T. gambiense*. *Journal of Protozoology* **28**:470-9.

Brun R, Schonenberger M. (1979)Cultivation and *in vitro* cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. *Acta Tropica* **36**:289-92.

Campbell GH, Esser KM, Phillips SM. (1978)*Trypanosoma rhodesiense* Infection in Congenitally Athymic (Nude) Mice. *Infection and Immunity* **20**:714-20.

Carruthers VB, Cross GAM. (1992)High Efficiency clonal growth of bloodstream- and insect-form *Trypanosoma brucei* on agarose plates. *Proceedings of The National Academy of Sciences of The United States of America* **89**:8818-21.

Charlab R, Blaineau C, Schechtman D, Barcinski A. (1990)Granulocyte-Macrophage Colony-Stimulating Factor is a Growth-Factor for Promastigotes of *Leishmania mexicana amazonensis*. *Journal of Protozoology* **37**:352-7.

Coppens I, Opperdoes FR, Courtoy PJ, Baudhuin P. (1987)Receptor-Mediated Endocytosis in the Bloodstream Form of *Trypanosoma brucei*. *Journal of Protozoology* **34**:465-73.

Crowe JS, Lamont AG, Barry JD, Vickerman K. (1984)Cytotoxicity of monoclonal antibodies to *Trypanosoma brucei*. *Transactions of The Royal Society of Tropical Medicine and Hygiene* **78**:508-13.

Cunningham I. (1986)Infectivity of *Trypanosoma rhodesiense* Cultivated at 28C with Various Tsetse Fly Tissues. *Journal of Protozoology* **33**:226-31.

Darji A, Beschin A, Sileghem M, Heremans H, Byrs L, De Baetselier P. (1996)*In Vitro* Simulation of Immunosuppression Caused by *Trypanosoma brucei*: Active Involvement of Gamma Interferon and Tumour Necrosis Factor in Pathway of Suppression. *Infection and Immunity* :1937-43.

Darji A, Sileghem M, Hermans H, Brys L, Baetselier P. (1993)Inhibition of T-cell Responsiveness during Experimental Infections with *Trypanosoma brucei*: Active Involvement of Endogenous Gamma Interferon. *Infection and Immunity* **61**:3098-102.

De Gee ALW, Mansfield JM. (1984)Genetics of Resistance to the African Trypanosomes Part IV. Resistance of Radiation Chimeras to *Trypanosoma rhodesiense* Infection. *Cellular Immunology* **87**:85-91.

De Gee ALW, Levine RF, Mansfield JM. (1988)Genetics of Resistance to the African Trypanosomes Part VI. Heredity of Resistance and Variable Surface Glycoprotein-Specific Immune Responses. *The Journal of Immunology* **140**:283-288.

Denis M. (1992)Interleukin-6 Is Used as a Growth Factor by Virulent *Mycobacterium avium*: Presence of Specific Receptors. *Cellular Immunology* **141**:182-8.

Ding AH, Nathan CF, Steubr D. (1988)Release of Reactive Nitrogen Intermediates and Reactive Oxgen Intermediates from Mouse Peritoneal Macrophages: Comparison of

Activating Cytokines and Evidence for Independent Production. *Journal of Immunology* **141**:2407-12.

Drapier JC, Pellat C, Henry Y. (1991)Generation of EPR-detectable Nitrosyl-Iron Complexes in Tumour Target cells Cocultured with Activated Macrophages. *Journal of Biological Chemistry* **266**:10161-7.

Duszenko M, Ferguson MAJ, Lamont GS, Rifkin MR, Cross GAM. (1985)Cysteine Eliminates the Feeder Cell Requirement for Cultivation of *Trypanosoma brucei* Bloodstream forms *in vitro*. *Journal of Experimental Medicine* **162**:1256-63.

Duszenko M, Muhlstadt K, Broder A. (1992)Cysteine is an essential growth factor for *Trypanosoma brucei* bloodstream forms. *Molecular and Biochemical Parasitology* **50**:269-74.

Fairlamb AH. (1989)Novel biochemical pathways in parasitic protozoa. *Parasitology* **99**:S93-S112.

Ferrante A, Allison AC. (1983)Alternative pathway activation of complement by African trypanosomes lacking a glycoprotein coat. *Parasite Immunology* **5**:491-8.

Flemming B, Diggs C. (1978)Antibody-Dependent Cytotoxicity against *Trypanosoma rhodesiense* mediated through an alternative complement pathway. *Infection and Immunity* 19:928-33.

Goretski J, Hollocher TC. (1988)Trapping of Nitric Oxide Produced during Denitrification by Extracellular Hemoglobin. *Journal Of Biological Chemistry* **263**:2316-23.

Green SJ, Meltzer MS, Hibbs JB, Nacy CA. (1990)Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. *Journal of Immunology* **144**:278-83.

Greenblatt HC, Rosenstreich DL. (1984)*Trypanosoma rhodesiense* Infection in Mice: Sex Dependence of Resistance. *Infection and Immunity* **43**:337-340.

Greenwood BM, Whittle HC. (1980)The Pathogenesis of sleeping sickness. *Transactions of The Royal Society of Tropical Medicine and Hygiene* **74**:715-25.

Grosskinsky CM, Askonas BA. (1981)Macrophages as Primary Target Cells and Mediators of Immune Dysfunction in African Trypanosomiasis. *Infection and Immunity* **33**:149-155.

Hamm B, Schindler A, Mecke D, Duszenko M. (1990)Differentiation of *Trypanosoma brucei* bloodstream trypomastigotes from long slender to short stumpy-like forms in axenic culture. *Molecular and Biochemical Parasitology* **40**:13-22.

Hart DT, Baudhuin P, Opperdoes FR, de Duve C. (1987)Biogenesis of the glycosome in *Trypanosoma brucei*: the synthesis, translocation and turnover of glycosomal polypeptides. *Embo Journal* **16**:1403-11.

Hecker H, Brun R. (1982)Comparative morphometric analysis of bloodstream and lymph forms of *Trypanosoma (T) brucei brucei* grown *in vitro* and *in vivo*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **76**:692-7.

Herbert WJ, Lumsden WHR. (1976)*Trypanosoma brucei*: A Rapid "Matching" Method for Estimating the Host's Parasitaemia. *Experimental Parasitology* **40**:427-31.

Hesse F, Selzer PM, Muhlstadt K, Duszenko M. (1995)A novel cultivation technique for long-term maintenance of blood-stream form trypanosomes *in-vitro*. *Molecular and Biochemical Parasitology* **70**:157-66.

Hibbs JB, Taintor RR, Vavrin Z. (1990)Synthesis of nitric oxide from a terminal guanidino nitrogen atom of L-arginine: a molecular mechanism regulating cellular proliferation that targets intracellular iron. In: Nitric oxide from L-arginine: a bioregulatory system. (Editors; Moncada S, Higgs EA). p189-223. Elsevier Science Publishers BV, Cambridge, New York.

Hide G, Gray A, Harrison CM, Tait A. (1989)Identification of an epidermal growth factor receptor homologue in trypanosomes. *Molecular and Biochemical Parasitology* **36**:51-60.

Hill GC, Shimer SP, Caughey B, Sauer S. (1978a)Growth of infective forms of *Trypanosoma (T.) brucei* on buffalo lung and Chinese hamster lung tissue culture cells. *Acta Tropica* **35**:201-7.

Hill GC, Shimer SP, Caughey B, Sauer S. (1978b)Growth of Infective forms of *Trypanosoma rhodesiense in vitro*, the Causative Agent of African Trypanosomiasis. *Science* **202**:763-5.

Hirumi H, Doyle JJ, Hirumi K. (1977a)Cultivation of bloodstream *Trypanosoma brucei*. *Bulletin of the WHO* **55**:405-9.

Hirumi H, Doyle JJ, Hirumi K. (1977b)African Trypanosomes: Cultivation of Animal Infective *Trypanosoma brucei in vitro*. *Science* **196**:992-4.

Hirumi H, Hirumi K. (1989)Continuous Culture of *Trypanosoma brucei* Blood Stream Forms in a Medium Containing a Low Concentration of Serum Protein Without Feeder Cell Layers. *Journal of Parasitology* **75**:985-9.

Hirumi H, Hirumi K, Moloo SK, Shaw MK. (1992)*Trypanosoma brucei brucei*: *In vitro* Production of Metacyclic Forms. *Journal of Protozoology* **39**:619-627.

Hirumi H, Hirumi K, Nelson RT, Bwayo JJ. (1980)Present Status of in vitro Cultivation of Animal-Infective African Trypanosomes. In:Tropical Disease Research Series No.3; The *in vitro* Cultivation of Pathogens of Tropical Diseases, p165-200 Schwabe & Co. AG, Basel.

Hodgkinson VH, Soong L, Duboise SM, McMahonpratt D. (1996)*Leishmania amazonensis* - cultivation and characterization of axenic amastigote-like organisms. *Experimental Parasitology* **83**:94-105.

Hudson KM, Byner C, Freeman J, Terry RJ. (1976)Immunodepression, high IgM levels and evasion of the immune response in murine trypanosomiasis. *Nature* **264**:256-7.

James SL. (1995)Role of Nitric Oxide in Parasitic Infections. *Microbiological Reviews* **59**:533-47.

Jayawardena AN, Waksman BH, Eardly DD. (1978)Activation of Distinct Helper and Suppressor T cells in Experimental Trypanosomiasis. *Journal of Immunology* **121**:622-8.

Jennings FW, Whitelaw DD, Holmes PH, Chizyuka HGA, Urquhart GM. (1979)The brain as a source of relapsing *Trypanosoma brucei* infection in mice after Chemotherapy. *International Journal for Parasitology* **9**:381-4.

Jia L, Bonaventura C, Bonaventura J, Stamler JS. (1996)S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. *Nature* **380**:221-6.

Johnston A, Thorpe R. (1982) Immunohistochemistry in Practice, (editors; Johnston A, Thorpe R), p78, Blackwell Scientific Publications, Oxford, London, Edinburgh, Boston, Melbourne.

Jones JF, Hancock GE. (1983)Trypanosomiasis in Mice with Naturally Occuring Immunodeficiencies. *Infection and Immunity* **42**:848-51.

Kaminsky R, Beaudoin E, Cunningham I. (1988)Cultivation of the life cycle stages of *Trypanosoma brucei* sspp. *Acta Tropica* **45**:33-43.

Kantor DB, Lanzrein M, Stary SJ, Sandoval GM, Smith WB, Sullivan BM, Davidson N, Shuman EM. (1996)A Role for Endothelial NO Synthase in LTP Revealed by Adenovirus-Mediated Inhibition and Rescue. *Science* **274**:1744-1748.

Kardami E, Pearson TW, Beecroft R, P., Fandrich RR. (1992)Identification of basic fibroblast growth factor-like proteins in African trypanosomes and Leishmania. *Molecular and Biochemical Parasitology* **51**:171-82.

Kongshavin PAL, Ghadirian E. (1988)Enhancing and suppressive effects of tumour necrosis factor/cachectin on growth of *Trypanosoma musculi*. *Parasite Immunology* **10**:581-8.

Kuzoe FAS. (1993)Current Situation of African Trypanosomiasis. *Acta Tropica* **54**:153-62.

Lambert PH, Berney M, Kazyumba G. (1981)Immune Complexes in Serum and in Cerebrospinal Fluid in African Trypanosomiasis. *Journal of Clinical Investigation* **67**:77-85.

Lancaster JR. (1994)Stimulation of the diffusion and reaction of endogenously produced nitric oxide. *Proceedings of The National Academy of Sciences of The United States of America* **91**:8137-41.

Lagermans JAM, Vanderhulst MEB, Nibbering PH, Vanfurth R. (1992)Endogenous tumour-necrosis-factor-alpha is required for enhanced antimicrobial activity against *Toxoplasma gondii* and *Listeria monocytogenes* in recombinant gamma interferon-treated mice. *Infection and Immunity* **60**:5107-5112.

Laveissiere C, Sane B, Meda HA. (1994)Measurement of risk in endemic areas of human African trypansomiasis in Cote d'Ivoire. *Transactions of The Royal Society of Tropical Medicine and Hygiene* **88**:645-8.

Le Page RWF. (1967)Short Term Cultivation of *Trypanosoma brucei in vitro* at 37C. *Nature* **216**:1141-2.

Levine RF, Mansfield JM. (1981)Genetics of Resistance to African Trypanosomes: Role of the H-2 Locus in Determining Resistance to Infection with *Trypanosoma rhodesiense*. *Infection and Immunity* **34**:513-8.

Liew FY, Cox FEG. (1991)Nonspecific Defense Mechanisms: the Role of Nitric Oxide. *Parasitology Today* **7**:A17-21.

Liew FY, Millot S, Parkinson C, Palmer RMJ, Moncada S. (1990)Macrophage killing of Leishmania parasite *in vivo* is mediated by nitric oxide from L-arginine. *Journal of Immunology* **144**:4794-7.

Lorenz P, Betschart B, Owen JS. (1995)*Trypanosoma-brucei-brucei* and high-density-lipoproteins - old and new thoughts on the identity and mechanism of the trypanocidal factor in human serum. *Parasitology Today* **11**:348-52.

Lowenstein CJ, Snyder SH. (1992)Nitric Oxide, A Novel Biological Messenger. *Cell* **70**:705-7.

Mabbot N, Sternberg J. (1995)Bone Marrow Nitric Oxide Production and Development of Anemia in *Trypanosoma brucei*-Infected Mice. *Infection and Immunity* **63**:1563-6.

Mabbot N, Sutherland I, Sternberg J. (1994)*Trypanosoma brucei* is protected from the cytostatic effects of nitric oxide under *in vivo* conditions. *Parasitology Research* **80**:687-90.

Mabbot N, Sutherland I, Sternberg J. (1995)Suppressor Macrophages in *Trypanosoma brucei* infection: nitric oxide is related to both suppressive activity and lifespan *in vivo*. *Parasite Immunology* **17**:143-150.

MacAskill JA, Holmes PH, Whitelaw DD, McConnell I, Jennings FW, Urquhart GM. (1980)Immunological clearance of ^{75}Se-labelled *Trypanosoma brucei* in mice. II. Mechanisms in immune animals. *Immunology* **40**: 629-635.

MacAskill JA, Holmes PH, Jennings FW, Urquhart GM. (1981) Immunological clearance of ^{75}Se-labelled *Trypanosoma brucei* in mice. III Studies in animals with Acute Infections. *Immunology* **43**: 691-698.

Magez S, Lucas R, Darji A, Songa EB, Hamers R, Baetselier P. (1993)Murine tumour necrosis factor plays a protective role during the initial phase of the experimental infection with *Trypanosoma brucei brucei*. *Parasite Immunology* **15**:635-641.

Marczin N, Jilling T, Papapetropoulous A, Go C, Catravas JD. (1996)Cytoskeleton-dependent activation of the inducible nitric oxide synthase in cultured aortic smooth muscle cells. *British Journal of Pharmacology* **118**:1085-94.

Matthews KR, Sherwin T, Gull K. (1995)Mitochondrial genome repositioning during the differentiation of the African trypanosome between life-cycle forms is microtubule mediated. *Journal of Cell Science* **108**:2231-9.

Mazingue C, Cottrez-Detoeuf F, Louis J, Kweider M, Auriault C, Capron A. (1989)*In vitro* and *in vivo* effects of interleukin 2 on the protozoan parasite *leishmania*. *European Journal of Immunology* **19**:487-91.

Mclintock LML, Turner CMR, Vickerman K. (1990)A Comparison of Multiplication Rates of Primary and Challenge Infections of *Trypanosoma brucei* Bloodstream forms. *Parasitology* **101**:49-55.

Mclintock LML, Turner CMR, Vickerman K. (1993)Comparison of the effects of immune killing mechanisms on *Trypanosoma brucei* parasites of slender and stumpy morphology. *Parasite Immunology* **15**:475-80.

Mills CD. (1991)Molecular Basis of "Suppressor" Macrophages. *Journal of Immunology* **146**:2719-23.

Milligan, K. (1996)Cell Cycle, Growth and Differentiation in *Trypanosoma brucei* and Leishmania species. Unpublished PhD Thesis, University of Glasgow.

Moncada S, Palmer RMJ, Higgs EA. (1991)Nitric Oxide: Physiology, Pathophysiology and Pharmacology. *Pharmacological Reviews* **43**:109-42.

Moreira MEC, Del Portillo HA, Milder RV, Balanco JMF, Barcinski MA. (1996)Heat Shock Induction of Apoptosis in Promastigotes of the Unicellular Organism *Leishmania (Leishmania) amazonensis*. *Journal of Cellular Physiology* **167**:305-313.

Nantulya VM, Musoke AJ, Rurangirwa FR, Barbet AF, Ngaira J, Katende JM. (1982)Immune Depression in African Trypanosomiasis:the role of Antigenic Competition. *Clinical and Experimental Immunology* **47**:234-42.

Napolitano LM, Campbell C. (1994)Nitric Oxide Inhibition Normalizes Splenocyte Interleukin-10 Synthesis in Murine Thermal Injury. *Archives of Surgery* **129**:1276-1283.

Newton BA. (1974)The chemotherapy of trypanosomiasis and leishmaniasis: towards a more rational approach. In: Trypanosomiasis and Leishmaniasis, 285-307. Elsevier/Excerpta Medica/North Holland, Amsterdam.

Olsson T, Bakhiet M, Edlund C, Hojeberg B, Van Der Meide P, Kristensson K. (1991)Bidirectional activating signals between *Trypanosoma brucei* and $CD8^+$ T cells: a trypanosome-released factor triggers Interferon-gamma production that stimulates parasite growth. *European Journal of Immunology* **21**:2447-54.

Olsson T, Bakhiet M, Hojeberg B, Ljungdahl A, Edlund C, Andersson G, Ekre H, Fung-Leung W, Mak T, Wigzell H, Fiszer U, Kristensson K. (1993)CD8 is Critically Involved in Lymphocyte Activation by a *Trypanosoma brucei brucei*-Released Molecule. *Cell* **72**:715-27.

Orago ASS, Facer CA. (1993)Cytokine-induced inhibition of *Plasmodium falciparum* erythrocytic growth *in vitro*. *Clinical and Experimental Immunology* **91**:287-94.

Oura CAL, Innes EA, Wastling JM, Entrican G, Panton WRM. (1993)The inhibitory effect of ovine recombinant interferon-gamma on intracellular replication of *Toxoplasma gondii*. *Parasite Immunology* **15**:535-8.

Poltera AA. (1980)Immunopathological and chemotherapeutic studies in experimental trypanosomiasis with special reference to the heart and brain. *Transactions Of The Royal Society of Tropical Medicine and Hygiene* 74:706-15.

Poltera AA. (1985)Pathology of Human African Trypanosomiasis with Reference to Experimental African Trypanosomiasis and Infections of the CNS. *British Medical Bulletin* 41:169-74.

Poltera AA, Cox JN, Owor R. (1976)Pancarditis affecting the conducting system and all valves in human African Trypanosomiasis. *British Heart Journal* **38**:827-37.

Porat R, Clark BD, Wolff SM, Dinarello CA. (1991)Enhancement of Growth of Virulent Strains of *Escherichia coli* by Interleukin-1. *Science* **254**:430-2.

Rajan TV, Porte P, Yates JA, Keefer L, Schultz LD. (1996)Role of Nitric Oxide in Host Defense Against an Extracellular Metazoan Parasite. *Brugia malayi*. *Infection and Immunity* 64:3351-3.

Raper J, Nussenweig V, Tomlinson S. (1996)The Main Lytic Factor of *Trypanosoma brucei brucei* in Normal Human Serum is Not High Density Lipoprotein. *Journal of Experimental Medicine* **183**:1023-9.

Reincke M, Allolio B, Petzke F, Heppner C, Mbulamberi D, Vollmer D, Winkelmann W, Chrousos GP. (1993)Thyroid dysfunction in African Trypanosomiasis: a possible role for inflammatory cytokines. *Clinical Endocrinology* 39:455-61.

Robins-Browne RM, Schneider J, Metz J. (1975)Thrombocytopenia in Trypanosomiasis. *American Journal of Tropical Medicine and Hygiene* 24:226-31.

Robinson DR, Sherwin T, Ploubidou A, Bayard EH, Gull K. (1995)Microtubule Polarity and Dynamics in the Control of Organelle Positioning, Segregation and Cytokinesis in the Trypanosome Cell Cycle. *Journal of Cell Biology* **128**:1163-72.

Rottenberg ME, Bakhiet M, Olsson T, Kristensson K, Mak T, Wigzell H, Orn A. (1993)Differential Susceptibilities of Mice genomically Deleted of CD4 and CD8 to Infections with *Trypanosoma cruzi* and *Trypanosoma brucei*. *Infection and Immunity* 61:5129-33.

Ruetten H, Southan GJ, Abate A, Thiemermann C. (1996)Attenuation of endotoxin-induced multiple organ dysfunction by 1-amino-2-hydroxy-guanidine, a potent inhibitor of inducible nitric oxide synthase. *British Journal of Pharmacology* **118**:261-270.

Rurangirwa FR, Musoke AJ, Nantulya VM, Nkonge C, Njuguna L, Mushi EZ, Karstad L, Grootenhuis J. (1986)Immune effector mechanisms involved in the control

of parasitaemia in *Trypanosoma brucei*-infected wildebeest (*Connochaetes taurinus*). *Immunology* **58**:231-237.

Sacco RE, Hagan M, Donelson JE, Lynch RG. (1994)B lymphocytes of Mice Display an Aberrant Activation Phenotype and Are Cell Cycle Arrested in G0/G1a During Acute Infection with *Trypanosoma brucei*. *Journal of Immunology* **153**:1714-23.

Sacks DL, Askonas BA. (1980)Trypanosome-induced suppression of anti-parasite responses during experimental African Trypanosomiasis. *European Journal of Immunology* **10**:971-4.

Sacks DL, Selkirk M, Ogilvie B, Askonas BA. (1980)Intrinsic immunosuppressive activity of different trypanosome strains varies with parasite virulence. *Nature* **283**:476-478.

Sato K, Miyakawa K, Takeya M, Hattori M, Yui Y, Sunamoto M, Ichimori Y, Ushio Y, Takahashi K. (1995)Immunohistochemical expression of inducible nitric oxide synthase (iNOS) in reversible endotoxic shock studied by a novel monoclonal antibody against rat iNOS. *Journal of Leukocyte Biology* **57**:36-44.

Schell D, Borowy NK, Overath P. (1991a)Transferrin is a growth factor for the bloodstream form of *Trypanosoma brucei*. *Parasitology Research* **77**:558-60.

Schell D, Evers R, Pries D, Ziegelbauer K, Kiefer H, Lottspeich F, Cornelissen AWCA, Overath P. (1991b)A transferrin-binding protein of *Trypanosoma brucei* is encoded by one of the genes in the variant surface glycoprotein gene site. *The EMBO Journal* **10**:1061-6.

Schleifer KW, Filutowicz H, Schopf LR, Mansfield M. (1993)Characterization of T helper Cell Responses to the Trypanosome Variant Surface Glycoprotein. *The Journal of Immunology* **150**:2910-2919.

Schliefer KW, Mansfield JM. (1993)Suppressor Macrophages in African Trypanosomiasis Inhibit T cell Proliferative Responses by Nitric Oxide and Prostaglandins. *The Journal of Immunology* **151**:5492-503.

Scott A, (1995)Drug-resistance and genetic exchange in *Trypanosoma brucei*. Unpublished thesis, University of Glasgow.

Seed JR. (1977)The Role of Immunoglobulins in Immunity to *Trypanosoma brucei* gambiense. *International Journal for Parasitology* **7**:55-60.

Seed JR, Cornille RL, Risby EL, Gam AA. (1969)The presence of agglutinating antibody in the IgM immunoglobulin fraction of rabbit antiserum during experimental African trypanosomiasis. *Parasitology* **59**:283-92.

Seed JR, Effron HG. (1973)Simultaneous presence of different antigenic populations of *Trypanosoma brucei gambiense* in *Microtus montanus*. *Parasitology* **66**:269-278.

Selkirk M, Wilkins SR, Ogilvie B, Platt-Mills TAE. (1983)*In vitro* induction of human helper cell activity by *Trypanosoma brucei*. *Clinical and Experimental Immunology* **52**:512-8.

Shapiro SZ, Naessens J, Liesegang B, Moloo SK, Magondu J. (1984)Analysis of flow cytometry of DNA synthesis during the life cycle of African trypanosomes. *Acta Tropica* **41**:313-323.

Sherwin T, Gull K. (1989)The cell division cycle of *Trypanosoma brucei brucei*: timing of event markers and cytoskeletal modulations. *Philosophical Transactions of the Royal Society of London* **B 323**:573-88.

Sileghem M, Darji A, Hamers SR, Van De Winkel M, Baetselier P. (1989)Dual role of macrophages in the supression of interleukin 2 production and interleukin 2 receptor expression in trypanosome-infected mice. *European Journal of Immunology* **19**:829-35.

Sileghem M, Naessens J. (1995)Are CD8 T cells involved in control of African trypanosomiasis in a natural host environment? *European Journal of Immunology* **25**:1965-71.

Simpson L. (1972)The kinetoplast of the hemoflagellates. *International Review of Cytology* **32**:139-207.

Ssenyonga GSZ, Adams KMG. (1975)The number and morphology of trypanosomes in the blood and lymph of rats infected with *Trypanosoma brucei* and *T. congolense*. *Parasitology* **70**:255-261.

Sternberg J, Mabbot N. (1996)Nitric oxide-mediated suppression of T cell responses during *Trypanosoma brucei* infection: soluble trypanosome products and interferon-gamma are synergistic inducers of nitric oxide synthase. *European Journal of Immunology* **26**:539-43.

Sternberg J, Mabbot N, Sutherland I, Liew FY. (1994)Inhibition of Nitric Oxide Synthesis Leads to Reduced Parasitaemia in Murine *Trypanosoma brucei* Infection. *Infection and Immunity* **May**:2135-7.

Sternberg J, McGuigan F. (1992)Nitric oxide mediates suppression of T cell responses in murine *Trypanosoma brucei* infection. *European Journal of Immunology* **22**:2741-4.

Sternberg J, McGuigan F. (1994)*Trypanosoma brucei*: Mammalian Epidermal Growth Factor Promotes the Growth of the AfricanTrypanosome Bloodstream Form. *Experimental Parasitology* **78**:422-4.

Sztein MB, Kierszenbaum F. (1991)A Soluble factor from *Trypanosoma brucei rhodesiense* that prevents progression of activated T lymphocytes through the cell cycle. *Immunology* **73**:180-5.

Tait A, Turner CMR. (1990)Genetic exchange in *Trypanosoma-brucei*. *Parasitology Today* **6**:70-5.

Tanner M. (1980)Studies on the mechanisms supporting the continuous growth of *Trypanosoma (Trypanozoon) brucei* as bloodstream-like forms *in vitro*. *Acta Tropica* **37**:203-20.

Taylor K, Lutje V, Mertens B. (1996)Nitric Oxide Synthesis is Depressed in *Bos indicus* Cattle Infected with *Trypanosoma congolense* and *Trypanosma vivax* and Does Not Mediate T-cell Suppression. *Infection And Immunity* **64**:4115-4122.

Turner CMR. (1990)The Use of Experimental Artifacts in African Trypanosomiasis Research. Parasitology Today **6**:14-17.

Turner CMR, Aslam N, Angus SD. (1996)Inhibition of growth of *Trypanosoma brucei* parasites in chronic infection. *Parasitology Research* **82**:61-66.

Turner CMR, Aslam N, Dye C. (1995)Replication, Differentiation, growth and the virulence of *Trypanosoma brucei* infections. *Parasitology* **111**:289-300.

Turner CMR, Barry JD, Vickerman K. (1988)Loss of variable antigen during transformation of *Trypanosoma brucei rhodesiense* from blood-stream to procyclic forms in the tsetse-fly. *Parasitology Research* **74**:507-11.

Turner CMR, Hunter CA, Barry JD, Vickerman K. (1986)Similarity in VAT composition of *Trypanosoma brucei rhodesiense* populations in different sites within the mouse host. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **80**:824-830.

Van Meirvenne N, Janssens PG, Magnus E. (1975)Antigenic variation in syringe passaged populations of *Trypanosoma (Trypanozoon) brucei*. I. Rationaliztion of the Experimental Approach. *Annals de la Societie de Belge Medecine Tropicale* **55**:1-23.

VanVelthuysen M, Mayen AEM, Rooijen N, Fleuren GJ, Heer E, Bruijn JA. (1994)T cells and Macrophages in *Trypanosoma brucei* related Glomerulopathy. *Infection and Immunity* **62**:3230-5.

Vassella E, Boshart M. (1996)High molecular weight agarose matrix supports growth of pleomorphic *Trypanosoma brucei* strains in axenic culture. *Molecular and Biochemical Parasitology* **82**:91-105.

Vickerman K. (1985)Developmental cycles and biology of pathogenic trypanosomes. *British Medical Bulletin* **41**:105-14.

Vickerman K, Myler PJ, Stuart KD. (1993), Afican Trypanosomiasis, In: Immunology and Molecular Biology of Parasitic Infections. 3rd edition p170-212, Blackwell Scientific Publications, Oxford.

Vincendeau P, Daulouede S. (1991)Macrophage Cytostatic Effect on *Trypanosoma musculi* involves an L-arginine - Dependent Mechanism. *The Journal of Immunology* **146**:4338-43.

Vincendeau P, Daulouede S, Veyret B, Darde ML, Bouteille B, Lemesre JL. (1992)Nitric Oxide - Mediated Cytostatic Activity on *Trypanosoma brucei gambiense* and *Trypanosoma brucei brucei*. *Experimental Parasitology* **75**:353-60.

Wang CC. (1995)Molecular Mechanisms and Therapeutic Approaches to the Treatment of African Trypanosomiasis. *Annual Review of Pharmacological Toxicology* **35**:93-127.

Wei X, Charles IG, Smith A, Ure J, Feng G, Huang F, Xu D, Muller W, Moncada S, Liew F. (1995)Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* **375**:408-411.

Welburn SC, Barcinski MA, Williams GT. (1997)Programmed Cell Death in Trypanosomiasis. *Parasitology Today* **13**:22-6.

Wharton M, Granger DL, Durack DT. (1988)Mitochondrial iron loss from leukemia cells injured by macrophages. *Journal of Immunology* **141**:1311-7.

Wijers DJB. (1960)Studies on the Behaviour of Trypanosomes belonging to the Brucei subgroup in the Mammalian Host. Unpublished Thesis, University of Amsterdam:p20-25.

Willson M, Callens M, Kuntz DA, Perie J, Opperdoes FR. (1993)Synthesis and activity of inhibitors highly specific for the glycolytic enzymes from *Trypanosoma brucei*. *Molecular And Biochemical Parasitology* **59**:201-10.

Wood J, Garthwaite J. (1994)Models of the Diffusional Spread of Nitric Oxide: Implications for Neural Nitric Oxide Signalling and its Pharmacological Proerties. *Neuropharmacology* **33**:1235-44.

Woodruff AW, Zeigler JL, Hathaway A, Gwata T. (1973)Communications: Anaemia in African Trypanosomiasis and Big Spleen Disease in Uganda. *Tropical Medicine and Hygiene* **167**:329-37.

Woodward R, Gull K. (1990)Timing of nuclear and kinetoplast DNA replication and early morphological events in the cell cycle of *Trypanosoma brucei*. *Journal of Cell Science* **95**:49-57.

Zaman V, Keong LA. (1990)Trypanosoma spp Infecting Man - African Trypanosomes. In: Handbook of Medical Parasitology 2nd ed, p40-44. Churchill Livingstone, London.