ACUTE PHASE RESPONSE AND THE POSSIBLE INVOLVEMENT OF AN ENDOTOXIN-LIKE MOLECULE IN THE PATHOGENESIS OF MURINE AFRICAN TRYPANOSOMIASIS

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ABSTRACT

This thesis describes a series of studies in mice infected with either *Trypanosoma brucei brucei* and *Trypanosoma congolense*, with the primary aim of identifying the pathogenic mechanism responsible for the acute phase response which occurs during African trypanosomiasis. The acute phase response was monitored by measuring the acute phase proteins, serum amyloid P-component (SAP) and haptoglobin (Hp) during a tissue invasive and non-tissue invasive infection. The possible involvement of trypanosome endotoxin in the pathogenesis of trypanosomiasis was also investigated, by measuring the endotoxin levels and acute phase proteins in plasma of infected animals during treatment with either a general, systemic antibiotic (norfloxacin) or one that is restricted to the intestinal tract and binds to and neutralises the pathogenic effects of endotoxin (polymyxin-B). In addition, the endotoxin-like molecule(s) in trypanosome lysates or enriched protein membranes were examined to determine the presence of lipopolysaccharide or the active moiety, lipid-A, using polyacrylamide gel electrophoresis and the silver stain, or Western blot utilising a lipid-A specific monoclonal antibody.

Chapter I comprises an introduction and literature review on African trypanosomiasis with emphasis on endotoxin, cytokines and acute phase proteins.

Chapter II describes the general materials and methods used in these studies.

Chapter III describes the development of a direct antigen enzyme linked immunosorbent assay (ELISA) for the quantification of mouse serum amyloid P-component.

Chapter IV describes the acute phase response during chronic murine trypanosome infection with tissue invasive and non-invasive trypanosomes, and
the effect of subcurative treatment with the trypanocidal drug, diminazine aceturate. It was found that the acute phase proteins SAP and haptoglobin increased significantly after infection. Following infection with *T. b. brucei*, SAP increased in both infections to peak 7-10 days after infection (DAI), after which it declined to levels just above control values where it remained for the rest of the infection period. In contrast, with *T. congolense* infection a second peak occurred around 34 DAI. The levels of SAP were not affected by the treatment with diminazine aceturate.

Haptoglobin (Hp) increased to peak at 7-10 DAI, and remained elevated throughout the infection in both experiments, but decreased significantly following treatment with diminazine aceturate in the *T. congolense* but not in the *T. brucei* infection where it remained elevated. The diminazine aceturate treatment in *T. brucei* infected mice resulted in severe cellular infiltration of mononuclear cells in the brains of these animals. As acute phase response occurs with both species of trypanosomes, it was concluded that tissue invasiveness is not the main means by which trypanosomes initiate tissue damage in infected hosts but that tissue pathology in the brain can cause synthesis of acute phase protein in the liver.

Chapter V describes the changes in plasma endotoxin-like activity, the concentration of the acute phase proteins, SAP and Hp, and tissue pathology during chronic *T. b. brucei* infections in mouse, the presence or absence of an antibiotic umbrella, with norfloxacin or polymyxin-B. The concentration of acute phase proteins and endotoxin-like activity increased significantly following infection. The animals also showed varying pathological changes during the different stages of infection. The spleen showed cellular activity, livers were markedly infiltrated with inflammatory cells with occasional necrosis, while the choroid plexus of the brains was infiltrated by trypanosome. Treatment with the antibiotic polymyxin-B, had no significant effect on any of
the parameters examined, whereas in the norfloxacin-treated animals there was a small but significant decrease in the haptoglobin levels in the terminal stages of infection after 21 DAI. The norfloxacin-treated animals also showed reduced liver pathology but only in the early stages of infection, i.e., before 21 DAI.

Chapter VI describes the characterisation of trypanosome lysate and protein-enriched membrane proteins by the silver stain and by Western blot. The results showed that trypanosomes do not contain a gram negative bacteria-like LPS molecule. On the other hand, the lipid-A monoclonal antibody (8A1) recognised epitopes on trypanosome lysate and protein-enriched membranes on Western blots; this were destroyed by pre-treatment of the samples with proteinase K, suggesting they are protein in nature or are associated with protein.

The general discussion and conclusions drawn from the study are presented in chapter VII. African trypanosomiasis causes an acute phase response and tissue damage probably by the production of pro-inflammatory cytokines which are induced probably by molecule(s) from trypanosomes with endotoxin-like activity. The molecule(s) responsible for the increased endotoxin-like activity is of trypanosome origin. Although this molecule(s) has lipid-A like activity and epitopes recognised by lipid-A monoclonal antibody, this trypanosome molecule(s), is dissimilar to the gram negative bacteria LPS.
DECLARATION

The work presented in this thesis is original and has been carried out solely by the author, except where collaboration with others has been acknowledged.

Raphael Muchangi Ngure
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DEDICATION

To my mum,

thank you for your encouragement, prayers and love.

To my dad,

I am sure you would have been happy and proud for all the effort you put into all this.

To my God Jehovah,
great is Thy faithfulness, great is Thy faithfulness o GOD my father, great is thy faithfulness, great is thy faithfulness, morning by morning new mercies I have seen; all I have needed Thy hand hath provided, great is Thy faithfulness, Lord, unto me.
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Silver stain SDS-PAGE gel loaded with LPS (L), Lipid-A (A), saline (S) and trypanosome lysate (TL). Figure 6.2a samples were not treated with PK prior to electrophoresis while in Figure 6.2b the samples were treated with 25 μg of PK for 60 min. Note the ladder-like staining of the *E. coli* LPS.

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ABBREVIATIONS

$\alpha$  Alpha
Am  Amps
$\beta$  Beta
$\gamma$  Gamma
$\mu$  Micro
$\mu$m  Micrometers
$\pm$  Plus or minus
$\mu$g  Microgram(s)
$\mu$g/ml  Microgram per millilitre
$\mu$l  Microliters
$\mu$l  Microliter
ACTH  Adrenocorticotrophic hormone
AEC  3-Amino-9-ethyl carbazole
APP  Acute phase protein
APR  Acute phase response
BBB  Blood brain barrier
BSA  Bovine serum albumin
bwt  Body weight
CNS  Central nervous system
CRP  C-reactive protein
CSF  Cerebrospinal fluid
DAI  Day after infection
DAT  Days after treatment
DEAE  Diethylaminoethyl cellulose
DIC  Disseminated intravascular coagulopathy
DMSO  Dimethyl sulfoxide
EDTA  Ethylene diamine tetra acetic acid
ELISA  Enzyme linked immunosorbent assay
EU    Endotoxin units
EU/ml Endotoxin units per milliliter
xg    Gravity
g     Grams
HAT   Human African trypanosomiasis
H&E   Haematoxylin and eosin
H₂O   Water
Hp    Haptoglobin
hr    Hours
HRP   Horseradish peroxidase
IFN   Interferon
IgG   Immunoglobulin G
IgM   Immunoglobulin M
IL    Interleukin
IL-1  Interleukin-1
IL-6  Interlukin-6
INF-γ Interferon gamma
ip    Intraperitoneal
IU    International units
Kg    Kilograms
l     Liters
LAL   Limulus amoebocyte lysate
LPS   Lipopolysaccharide
M     Molar
mg/kg Milligram per kilogram
Min  Minutes
ml   Millilitres
mM  Millimolar
ng  Nanogram
°C  Degree centgrade/Celsius
OD  Optical density
PBS Phosphate buffered saline
PBST Phosphate buffered saline-Tween
PCV Packed red cell volume
pg  Picogram
SAA Serum amyloid A
SAP Serum amyloid P-component
SD  Standard deviation
SDS Sodium dodecyl sulphate
SDS- SDS-polyacrylamide gel electrophoresis
PAGE
Sec  Seconds
SEM Standard error of the mean
TBS Tri-buffered saline
TEMED N'N'N',-tetramethylenediamine
TLTF Trypanosome lymphocyte triggering factor
TMB Tetra methyl benzidine
ANOVA Analysis of variance
TNF Tumour necrosis factor
TNF-α Tumour necrosis factor alpha
TNF-β Tumour necrosis factor beta
v/v Volume per volume
w/v Weight per volume
CHAPTER 1
INTRODUCTION AND GENERAL LITERATURE REVIEW
1.1. INTRODUCTION

African trypanosomiasis is an economically important haemoprotozoan disease affecting man and his domestic animals. In the animal infection, millions of dollars are lost annually through death, reduction in productivity of meat and milk, and reduced fertility (Luckins, 1992; Sekoni, 1992). In humans, infection reduces the productivity of individuals and also causes a great constraint on already limited health budgets of third world countries due to the high cost of drugs for treatment and control of the disease. The infestation of vast areas of land by tsetse flies, the principal vector, also limits the maximum utilisation of land in the tropics due to the risk of the infection both to man and his animals.

The development of an appropriate vaccine against the trypanosome parasite has remained elusive due to the parasite's ability to escape the host immune system by changing its surface coat by the phenomenon of antigenic variation (Vickerman, 1978; Nantulya, 1986). Control strategy of human African trypanosomiasis (HAT) is thus dependent on continuous surveillance, systematic case detection and treatment, and the control of the vectors, which are mainly of the *Glossina* species (de Raadt, 1984; Kuzoe, 1989). These control strategies however have several constraints. Few new drugs have been introduced over the last few decades due to the expense involved in drug development, registration and also a perceived small human commercial market. This has resulted in reluctance of the drug industry to invest in the development of new trypanocides, and has led to the widespread continuous use of the few available drugs in field clinics over many years, resulting in drug resistance (Anon, 1991; Bacchi, 1993). Most of the drugs available do not cross the blood-brain barrier (BBB) and can only be used in the treatment of the early stage trypanosomiasis. The available drugs that can cross the BBB, and thus used for the treatment of late stage trypanosomiasis, include the arsenicals,
which are toxic and have adverse side effects (Adams, Haller, Boa, Doua, Dago and Konian, 1986). Another factor hindering effective treatment is the lack of easily affordable and sensitive diagnostic tests to enable early diagnosis and effective treatment before central nervous system (CNS) infection. This is compounded by the fact that the disease, being a rural condition, does not allow early reporting of cases. Thus most cases are diagnosed in the advanced stage with CNS involvement and are more difficult to treat. In human trypanosomiasis, painful diagnostic procedures, including lumber puncture, do not encourage the completion of therapeutic regimens and hinder the follow-up of patients after treatment (Anon, 1991).

Control of the tsetse fly is difficult due to the vast areas affected and lack of economically viable control methods. Also, effective control measures have been hampered by war and financial reasons, leading to re-invasion of areas that had been cleared of tsetse flies.

1.2. CLASSIFICATION OF TRYPANOSOMES

The pathogenic trypanosomes in the genus Trypanosoma, can be subdivided into two main groups, the stercorarian and salivarian (Table 1.1). This depends on the site of their development in the vector, and hence the mode of transmission. The stercorarian group develop in the alimentary tract, with the production of infective metacyclics occurring in the hind gut of the vector with the host infection either through skin contamination or by oral ingestion. *Trypanosoma cruzi*, the causative agent of Chagas' disease in South and Central America, is the most important pathogen of the group. On the other hand, the salivarian group complete their development in the anterior station, i.e., the salivary gland and the proboscis of tsetse fly, with the result that the parasites are transmitted by inoculation when the fly takes a blood meal on its host. The Brucei sub-group including *T. brucei brucei*, *T. b. gambiense*, and *T. b.*
rhodesiense are among the important pathogens of the salivarian group affecting man and animals (Stephen, 1986).

1.3. DISEASE IN MAN

1.3.1. Pathogenesis And Pathology

Human African trypanosomiasis can be divided geographically into the west African (gambiense) and the east African form (rhodesiense), caused by Trypanosoma brucei gambiense and T. brucei rhodesiense respectively.

The disease affects all body systems, including the central nervous system (CNS). It is characterised by an initial trypanosome multiplication in the subcutaneous tissue at the point of tsetse fly bite (Fairbairn and Godfrey, 1957; 1958). The tsetse fly inoculates the host with trypanosomes in saliva into the subcutaneous pool of blood during the process of feeding. Some of the organisms may enter directly into the blood stream (Willett and Gordon, 1957), but the majority get entangled in the extravascular tissue where they multiply by binary fission as long slender trypomastigotes, producing a local inflammatory reaction called a chancre (Fairbairn and Godfrey, 1957). The chancre is presented as a staphylococcal-like cellulitis characterised by a hard, painful, red nodule with marked oedema and erythema that can be several centimetres in diameter. It is more common in T. b. rhodesiense infections and in patients with a lighter skin (Molyneux, de Raadt and Seed, 1984). The trypanosomes then spread through the tissue spaces into the lymphatic system hence the local and general lymph nodes, where they continue to multiply. Once in the blood stream, the trypanosomes traverse the walls of blood and lymph capillaries and enter into tissues, including the CNS (Fairbairn and Godfrey, 1957; 1958).

For the tissue-invasive trypanosomes, i.e., the brucei group, invasion into most organs results in metabolic and biochemical changes, possibly caused
by direct parasite action or indirectly by host immune response to the parasite. If the human survives the acute phase of infection, the trypanosomes then progressively invade the CNS resulting in a meningoencephalitis accompanied by neurological signs. The severity and time course of the trypanosome infection varies with species of trypanosomes involved (Losos and Ikede, 1973a). Typical *T. b. rhodesiense* infection, presents as an acute to subacute disease, accompanied by rapid development of severe parasitaemia, anaemia and acute cardiac abnormality leading to cardiac failure (Manson-Bahr and Charters, 1963; Manuelidis, Robertson, Amberson, Pola and Haymaker, 1965; Francis, 1972; Jones, Lowenthal and Buyst, 1975; Harries and Wirima, 1988). During this acute syndrome, neurological involvement is minimal since death occurs within weeks or a few months in untreated patients. On the other hand, *T. b. gambiense* infections present a subacute to chronic disease with slow heart involvement (Adams *et al*, 1986). It is characterised by low parasitaemia, marked progressive loss of body condition, and a predominant progressive neurological syndrome (Molyneux *et al*, 1984; Boa, Traore, Doua, Kouassi-Troure, Kouassi and Giodano, 1988). Atypical cases of chronic *T. b. rhodesiense* and acute *T. b. gambiense* infections have also been documented (Apted, 1970a).

The mechanisms that lead to the severe pathology of human African trypanosomiasis are still poorly understood, but appear to be related to the invasive capacity of the trypanosomes, their ability to generate biologically active mediators, and/or to stimulate immunological reactions by the host to this highly antigenic parasite which undergoes persistent antigenic variation (Borst and Cross, 1982; Steinert and Pays, 1985). The precise form of the human and animal trypanosomiasis, depends not only upon the host, and the species of infecting trypanosomes, but also upon the particular strain of the trypanosome involved (Losos and Ikede, 1973a; 1973b).
1.3.2. Clinical Signs

Gambian human sleeping sickness is characterised by a relatively long incubation period followed by a gradual but progressive onset of irregular fever, anaemia, splenomegaly, hepatomegaly, lymphadenopathy and cachexia (Apted, 1970a; Ormerod, 1970). Affected individuals eventually show neurological disturbances, including headache, changes in behaviour, and reversed sleep pattern manifested as somnolence during the day and insomnia at night (Greenwood and Whittle, 1980). Sensory symptoms are also frequent with cutaneous hyperaesthesia and deep hyperpathia (Boa et al, 1988), accompanied by changes in mental performance, lassitude, and general weakness. As the disease progresses, there is wasting and a gradual loss in the control of limbs, abdominal, and thoracic muscle, leading to coma, and death in untreated cases (Haller, Adams, Merouze and Dago, 1986). Signs of cardiac involvement are also encountered (Adams et al, 1986), but the disease is mainly associated with central CNS involvement (Molyneux et al, 1984; Boa et al, 1988). In its milder form, the progression of this disease may be extremely slow, and mortality may be correspondingly slow (Tizard, Nielsen, Seed and Hall, 1978).

In contrast T. b. rhodesiense, is a highly acute and fatal disease if untreated. The major clinical features include rapid onset of high undulating fever following each peak of parasitaemia. This is accompanied by headache, general weakness, and exercise intolerance. Anaemia is a consistent finding in T. b. rhodesiense infection resulting in development of pale mucous membranes (Woodruff, Ziegler, Hathaway and Gwata, 1973). In the rhodesiense infection, the central nervous signs are not dramatic, but some individuals may show evidence of an acute myocarditis accompanied by electrocardiographic abnormalities, including, heart blocks and murmurs of varying degree (Ormerod, 1970; Jones et al, 1975). Affected individuals suffer from
overwhelming parasitaemia, and death may occur within a few weeks or months (Apted, 1970a). On physical examination the lymph nodes, spleen and the liver are enlarged and easily palpable. Auscultation of the heart reveals cardiac abnormalities varying from tachycardia, murmurs and heart blocks. Oedema mainly of the lower limbs and occasionally affecting the face has been documented (Wellde, Chumo, Reardon, Mwangi, Asenti, Mbabi, Abinya, Wanyama and Smith, 1989), giving the moonface syndrome (Manson-Bahr and Charters, 1963).

Reproductive changes observed in human patients, include, abortion, amenorrhoea and impotence (Greenwood and Whittle, 1980), gynacomastia with feminine-like fat distribution in males (Molyneux et al, 1984; Boersma, Hublart, Boutignon, Noireau, Lemesre, O-Herbomez and Degand, 1989). Severe diarrhoea has also been observed (Basson, Page and Myburgh, 1977).

1.3.3. Pathology

Lymphoadenopathy and splenomegaly are common features in human and animal trypanosomiasis (Sadun, Johnson, Nagle and Duxbury, 1973). Lymph nodes are enlarged, flabby, oedematous, and sometimes haemorrhagic. This is accompanied by cellular proliferation in the lymphoid tissues consisting of large pyroninophilic lymphoid cell and plasma cells with occasional macrophages. In chronic cases, the lymph node plasma cells occupy the entire node except the lymphocytic follicles which sometimes contain active germinal centres while others are small and inactive. In the most advanced cases, capsular and trabecular fibrosis is evident (Ormerod, 1970). Similar changes occur in the spleen where the plasma cell response, expands and disrupts the white pulp including the thymic-dependent periarteriolar lymphocytic sheath and a large part of the red pulp. In the later stages of infection both the lymph nodes and spleen become depleted of immunological cell types exposing the
underlying framework of reticulum cells and fibroblast or alternatively histiocyte-like cells and multinucleated giant cells become prominent with occasional Mott cell appearance (Low and Mott, 1904). At the same time the lymph node and spleen in chronic cases atrophy (Ormerod, 1970).

The heart involvement is a common occurrence especially in the acute stages of *T. b. rhodesiense* infection and most acute deaths result from cardiac failure (Hawking and Greenfield, 1941; Manuelidis *et al*, 1965). At post-mortem examination, the heart is enlarged, soft and flabby, with effusions in the pericardium, pleura, and the peritoneal serous cavities (Manson-Bahr and Charter, 1963; Francis, 1972; Mbala, Blacket, Mbonifor, Leke and Etoundi, 1988). The serous fluid varies in colour and content, but is rich in plasma proteins and often contains trypanosomes (Hawking and Greenfield, 1941). Histologically, cardiac involvement presents as a pancarditis affecting all the heart layers including valves, draining blood and lymphatic vessels, and the conducting system (Poltera, Cox and Owor, 1976). These changes are the basis for the clinically observed cardiac insufficiency and electrocardiographic changes reported in infected patients (Jones *et al*, 1975; Poltera and Cox, 1977; Mbala *et al*, 1988). Microscopically, there is focal or diffuse mononuclear cell infiltration of the interstitial tissue and the perivascular spaces, accompanied by myocytolysis and focal endocardial fibrosis of the conducting system (Hawking and Greenfield, 1941; Poltera *et al*, 1976). Occlusion of the local draining lymphatics due to cellular infiltration, and phlebitis are common occurrences.

The livers from infected patients are usually enlarged, congested, mottled and pale with varying degree of fatty changes (Jenkins and Robertson, 1959; Apted, 1970a). Histologically there is an increased level of phagocytic cell activity and numbers, with enlargement of Kupffer cell and prominence of mitotic figures. Pneumonia is a common feature of trypanosomiasis accompanied by congestion and oedema of the lungs resulting from the
pancarditis, and/or secondary bacterial infection (Cohen, 1973; Poltera, Owor and Cox, 1977). This can be accompanied by pleuritis (Adams et al, 1986).

Ocular changes have been described in untreated human patients and include keratitis, iridocyclitis, retinitis, papilo-oedema, optic and atrophic neuritis (Greenwood and Whittle, 1980). In dogs varying degree of hypopyon, hyphema, and corneal opacity have been documented (Morrison, Murray, Sayer and Preston, 1981a).

The CNS involvement is the predominant sign of typical T. b. gambiense and chronic T. b. rhodesiense infection (Haller et al, 1986; Pentreath, 1989). The CNS shows minimal gross pathological changes during the acute phase of trypanosomiasis, but in the chronic cases there is thickening and congestion of the meninges (Greenwood, Whittle, Oduloju and Dourmaskin, 1976). Occasional purulent meningitis and brain oedema have been reported. On histological examination, brains of trypanosome-infected patients, present a non specific lymphocytic plasmacytic meningoencephalitis of varying intensity (Adams et al, 1986). The most striking feature is a perivascular infiltration of the Virchow-Robin spaces, i.e., perivascular cuffing by inflammatory cells mainly lymphocytes, plasma cells and in the advanced cases, macrophages. In these advanced cases, the inflammatory cells can be found extending into the neuropil with damage to the piaglial lining. These changes are most evident in the white matter of the cerebral hemispheres and cerebellum, the basal nuclei, and the brain stem, although similar lesions are seen in the deep layers of the cerebral cortex. Present also are Mott cells filled with immunoglobulins found at the perivascular space in association with other inflammatory cells or alone scattered in the neuropil. Diffuse microglial hyperplasia in the gray matter, and large reactive astrocytes throughout the white matter have been described in humans (Adams et al, 1986) and in experimental animals (Hunter, Jennings,
Kennedy and Murray, 1992a). Inflammatory cells are also found in the ventricles and the choroid plexus alone or in association with trypanosomes.

Accompanying CNS pathology, are changes in the cerebrospinal fluid (CSF). Normal human CSF is usually clear, containing less than 5 white blood cells (WBC) per ml and a protein content of lower than 0.25 gm per litre (Abaru, Liwo, Isakina and Okori, 1984). Immunoglobulin levels are also low with undetectable IgM immunoglobulins. In patients with advanced trypanosomiasis, trypanosome invade the CSF causing cellular and immunological changes that are of pathological and diagnostic importance. There is cellular infiltration of the CSF giving it a tinge of cloudiness and viscosity. These cells are mainly of the lymphocytes and plasma cell series, but with progress of the infection, Mott cells and macrophages appear. Chronic infection, especially with \textit{T. b. gambiense}, leads to a rapid CSF hypergammaglobulinaemia dominated by an increase in IgM immunoglobulins (Greenwood and Whittle, 1973). This results from an \textit{in situ} production of the immunoglobulins by the infiltrating plasma cells. The raised CSF IgM is of diagnostic importance in chronic \textit{T. b. gambiense} infections (Mattern, Klein, Radema and Van Furth, 1967). Accompanying the increase in immunoglobulins is the appearance of immune complexes in the serum and cerebrospinal fluid (Lambert, Berney and Kazyumba, 1981).

\textbf{1.4. POSSIBLE PATHOGENIC MECHANISM OF TRYPANOSOMIASIS}

The precise mechanism leading to the production of the many pathological alterations accompanying African trypanosomiasis is not yet clearly understood (Murray, 1974; Urquhart, 1980; Morrison, Murray, Sayer and Preston, 1981a; 1981b; Pentreath, 1991). The major changes that occur, include, anaemia, lymphoid proliferation, immunosuppression, increased capillary permeability and circulatory disturbances, tissue inflammation accompanied by severe tissue
damage, biochemical alterations and hormonal imbalances. Several hypothesis have been proposed to explain the mechanisms responsible for these pathological alterations. These include the possible presence of a trypanosome toxin, induction of pharmacological active products, release of enzyme from host tissues and disintegrating trypanosome and immune-mediated reaction with increased production of cytokines.

1.4.1. Toxin.

There has been speculation on the possible presence of a trypanosome toxin believed to be responsible for initiating the observed pathological changes during infection (Huan, Webb, Lambert and Meischer, 1975; Tizard et al, 1978). Seed (1969) isolated a protein fraction from the homogenates of *T. b. gambiense* which increased vascular permeability in rabbits while Murray, (1979) identified a haemolytic factor that may be operative *in vivo* during bovine trypanosomiasis (Murray, Huan, Lambert and Gerber, 1977). Indeed some of the pathological alteration of trypanosome infection can be produced by trypanosome lysate product, including, immunosuppression, mitogenic suppression (Clayton, Sacks, Ogilvie and Askonas, 1979), and increased vascular permeability. Recent work has demonstrated increased levels of endotoxin-like activity in serum of trypanosome infected mice and also in trypanosome lysates (Alafiayato, Crawley, Oppenheim and Pentreath, 1993). Trypanosome product(s) have been shown to block cell growth, inhibit the complete development of T-lymphocytes through the cell cycle (Sztein and Kierszenbaum, 1991) and growth of human promyelocytic cell types (Temitope, Seed, Sechelski and Balber, 1993). The precise nature of this toxin has not been determined but it could be the trypanosome molecule(s) responsible for initiating the production of the polyclonal-B cell activation (Urquhart, Murray, Murray, Jennings and Bate, 1973; Greenwood, 1974),
immunosuppression (Hudson, Byner, Freeman and Terry, 1976), and cytokine production (Reincke, Allolio, Petzke, Heppner, Mbulamberi, Vollmer, Winkelmann and Chrousos, 1993) during trypanosome infection. A trypanosome lymphocyte triggering factor (TLTF) has been purified from trypanosomes (Bakhiet, Olsson, Edlund, Hojeberg, Holmberg, Lorentzen and Kristensson, 1993). The TLTF was shown to be able to stimulate rat and human mononuclear cells to release interferon-γ (IFN-γ), an effect that was CD8⁺ T-lymphocyte dependent (Olsson, Bakhiet, Edlund, Hojeberg, van der Meide and Kristensson, 1991).

1.4.2. Induction of Harmful Metabolic Products

Trypanosomes have been shown to be able to metabolise aromatic amino acids to a series of potentially toxic products (Stibbs and Seed, 1973; 1975; 1976). Tryptophan is catabolised to indole-pyruvate, indole-lactate, indole acetate, and indole-ethanol. Tyrosine is metabolised to \( p \)-hydroxyphenylpyruvate and \( p \)-hydroxyphenyllactate while phenylalanine is catabolised to phenylpyruvate. Several of these compounds injected at pharmacological doses have been shown to have toxic properties. Indole-ethanol can induce a sleep-like state, alter body temperature (Seed and Sechelski, 1977), and be immunosuppressive (Sachez, Lockwood and Chavez, 1981).

1.4.3. Immunopathogenesis

Trypanosome infection in humans and animals results in both quantitative and qualitative changes in the immune system of the affected host. There is a decreased response in antibody production and mitogenic response to both trypanosome and non-trypanosome antigens, and increased
immunosuppression accompanied by elevated non-specific antibody production.

1.4.3.1. Changes in the lymphoid organs.

The lymphoid system in the trypanosome-infected host undergoes cellular and architectural changes that could undermine its effective function. There is normally enlargement of the spleen, and lymph nodes associated with marked cellular hyperplasia of the lymphocyte and mononuclear cells. This hyperplasia results in increased B-cell areas with increased large lymphoid cells and immature and mature plasma cells, while the periarteriolar lymphatic sheath (T-cell area) becomes depleted of small lymphocytes and is infiltrated with macrophages and plasmablasts (Murray Jennings, Murray and Urquhart, 1974b).

1.4.3.2. Immune complexes

Immune complexes can increase to high levels in serum and CSF of infected humans. This has been found to correlate well with polyclonal B cell activation and with intracerebral immunoglobulin synthesis (Lambert et al, 1981). The immune complexes are deposited in tissues and could lead to complement activation, disseminated intravascular coagulation, immune-mediated tissue damage, including vasculitis (Poltera, Hochmann, Rudin and Lambert, 1980a; Van Marck, Mulumba, Gigase and Wery, 1983), glomerulonephritis (Lambert and Houba, 1974; Sadun et al, 1973) and cardiomyopathy (Poltera, Hochmann and Lambert, 1980b), which are common alterations observed in trypanosomiasis. Similarly immune complex-deposition in tissues invaded by trypanosomes has been reported to occur in both rodents (Murray, 1974; Murray, Lambert and Morrison, 1975) and primates experimentally infected with African trypanosomes (Poltera, 1980).
1.4.3.3. Autoimmunity

Auto-antibody production is a feature of sleeping sickness. Auto-antibodies are produced against a wide range of antigens such as erythrocytes, smooth and striated muscles, and deoxyribonucleic acid (Kobayakawa, Louis, Izui and Lambert, 1979; Poltera et al, 1980a; Kazyumba, Berney, Brighouse, Cruchaud and Lambert, 1986). Other studies have found in human sera, the presence of high titers of antibody against brain myelin proteins (Ansonganyi, Lando and Ngu, 1989), and against galactocerebrosides, a major component of myelin (Amevigbe, Jauberteau-Marchan, Bouteille, Doua, Breton, Nicolas and Dumas, 1992). Immunoreactivity against galactocerebrosides has been demonstrated in trypanosome-infected sheep (Jauberteau, Younes-Chennoufi, Amevigbe, Bouteille, Dumas, Breton and Baumann, 1991). In infected mice, a strong correlation exists between the levels of auto-antibodies to myelin basic protein, galactocerebrosides and gangliosides, and damage to the CNS caused by the progression of infection or by the enhanced reactive damage following drug treatment (Hunter, Jennings, Tierney, Murray and Kennedy, 1992b). This observations suggests the possible production of the tissue pathology through an autoimmune-mediated mechanism.

1.4.4. Pharmacological Active Products
1.4.4.1. Autacoids

These are pharmacologically active products derived from inflammatory cells and injured tissue and, if released in high amounts into the circulation, can result in a wide effect on the host homeostatic mechanism. They are known to cause several effects, including, hypotension, increased capillary permeability, and be chemotactic (Ackerman and Seed, 1976; Boreham, 1978). During human and animal trypanosome infections, several autacoids have been shown
to increase significantly in the circulatory system, including, kinin, histamine, prostaglandin and 5-hydroxytryptamine (Richard, 1965; Boreman, 1970; Pentreath, Rees, Owolabi, Philip and Doua, 1990). These might be responsible for the observed cardiovascular disruption and changes in sleep patterns observed during trypanosomiasis (Pentreath et al, 1990). Increased prostaglandin levels in CSF in humans are thought to be responsible for the alteration in the sleep pattern. Infected rabbits (Stibbs, 1984), and mice (Stibbs and Curtis, 1987; Amole, Sharpless, Wittner and Tanowitz, 1989), also show decreased levels of neurochemical molecules in brain tissue which could explain the observed behavioural symptoms in infected hosts.

1.4.5. Enzymes

1.4.5.1. Trypanosome enzymes

Several trypanosome species have been shown to contain phospholipase A₁ and lysophosphalipase, with *T. b. brucei* having the highest levels (Mellor, 1985). Accumulated high levels have been recorded in tissue fluid of *T. b. brucei*-infected rabbits (Hambrey, Tizard and Mellor, 1980). This phospholipase could lead to the generation of membrane-active products from phospholipids such as free fatty acids, which are known to be immunosuppressive (Mertin and Hughes, 1975).

Plasma of mice infected with *T. b. brucei*, contains increased levels of parasite derived peptidase enzyme (Knowles, Black and Whitelaw, 1987). The presence of this enzymes might contribute to the increased catabolism of serum proteins reported by Jennings, Murray, Murray and Urquhart (1973).

1.4.5.2. Lysosomal enzymes

Phagocytic cells, mainly of the mononuclear series, are known to accumulate in the vicinity of tissues and blood vessels during trypanosomiasis
(Goodwin, 1971). These cells contain lysosomal enzymes which can be released with or without cell destruction, and which have the potential to destroy cell components and organelles. In trypanosomiasis, they could participate in the pathogenesis by increasing leakage from blood vessels by direct action on the vascular endothelium, by activating the kallikrein-kinin system, or by acting as chemotactic agents for granulocytes and destroying the connective tissue, features observed during trypanosome infection.

1.4.6. Other Reactive Molecules

Macrophages from trypanosome-infected hosts have been shown to produce high levels of nitric oxide and prostaglandin (PGE$_2$) that are speculated to be responsible for the inhibition of T-cell lymphocyte proliferation and reduced response to mitogen and trypanosome antigen (Sternberg and McGuigan, 1992; Schleifer and Mansfield, 1993; Mabbot, Sutherland and Sternberg, 1995). This might lead to the immunosuppression observed during trypanosomiasis, eventually resulting in secondary infections which are responsible for many deaths in chronic infections.

1.5. CLINICAL PATHOLOGY

1.5.1. Role of Cytokines in Disease and Trypanosomiasis

Cytokines are regulatory peptides that are produced by virtually every nucleated cell type, including monocytes, lymphocytes, vascular endothelium and smooth muscle cells, fibroblasts and hepatocytes (Andus, Bauer and Gerok, 1991).

Cytokines have pleiotropic regulatory effects on haemopoietic and non-haemopoietic cell types, which participate in the host defence and tissue repair processes. Monocytes and macrophages are the major source of most cytokines. Cytokines are extremely potent with autocrine and/or paracrine effects
producing their action through binding to specific high affinity cell surface receptors on the target cell. A number of important physiological functions have been attributed to cytokines (Balkwill and Burk, 1989; Andus et al., 1991). For example, they form a complex communication network among cells of the haematopoietic system in which they control the differentiation and maturation of stem cells in the bone marrow (Thomas, Reasor and Wierda, 1989). Cytokines involved in haematopoiesis, include, interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-7 (IL-7) and colony-stimulating factors (CSFs).

Cytokines also contribute to both the specific and non-specific immune response by regulating lymphocyte proliferation, differentiation and activation, and by increasing resistance to viral infection and neoplasms (O'Garra, 1989a; 1989b; Aiello, Longo, Overton, Takacs and Durum, 1990; Akira, Hirano, Taga and Kishimoto, 1990). Cytokines which regulate the immune system, include, interleukins, TNF-α, and interferons (Duff and Oppenheim, 1992; Akira et al., 1990). Some cytokines, including, IL-1, IL-6 and TNF-α, decrease the synthesis of plasma proteins, such as albumin, and increase the synthesis of the acute phase proteins (Andus et al., 1991).

Although cytokines perform beneficial regulatory functions in the haematopoietic and immune system, and during inflammation, evidence from numerous studies suggests that they may be involved in the development of tissue injury under certain pathological disease conditions (Titus, Sherry and Cerami, 1991), including the induction of inflammatory changes (Grau, Fajardo, Piguet, Allet, Lambert and Vasalli, 1987; Waage, Halstensen, Shalaby, Brandtzaeg, Kierulf and Espevik 1989; Ramilo, Saez-Llorens, Jafari, Olsen, Hansen, Yoshinaga, Ohkawara, Nariuchi and McCracken, 1990; Saukkonen, Sande, Cioffe, Wolpe, Sherry, Cerami and Tuomanen, 1990), fever (Tewari, Buhles and Starnes, 1990; Kluger, 1991; Nakamori, Morimoto, Yamaguchi,
Watanabe and Murakami, 1994), hypotension (Baldwin, Alpert, Caputo, Baskin, Parsonnet, Gillis, Thompson, Siber and Fleisher, 1991), and the production of hepatic acute phase proteins (Baumann and Gauldie, 1994). The extravasation of inflammatory cells to produce tissue inflammation is effected by cytokines through regulation of the production of adhesion molecules, including, intercellular adhesion molecule (ICAM) and endothelial adhesion molecule (ELAM) and also by the chemotactic effects of cytokines (Beutler and Cerami, 1988; Rees, 1991).

In the immune system, cytokines are potent regulators of both T and B cells growth and differentiation (Vilcek and Le, 1994). Cytokines are also known to activate macrophages by inducing macrophage differentiation and enhancing major histocompatibility complex (MHC) expression (Mackenzie, Culpepper, de Waal Malefyt, Briere, Punnonen, Aversa, Sato, Dang, Cocks, Menon, de Vries, Banchereau and Zurawski, 1993). In addition, they cause changes in the endothelial cell by production of adhesion molecules and also increasing the vascular permeability, thus, causing transduction of inflammatory cell into the extravascular spaces and tissues (Sharief, Ciardi and Thompson, 1992).

During trypanosomiasis increased levels of cytokines have been reported (Bancroft, Sutton, Morris and Askonas, 1983; Askonas and Bancroft, 1984; Le and Vilcek, 1987; Hunter, Gow, Kennedy, Jennings and Murray, 1991; Hunter et al, 1992a; Reinke et al, 1993) and may be important factors in the production of most of the observed pathological changes. They are thought to be responsible for the initiation of inflammatory changes in the CNS (Hunter et al, 1991; Hunter et al, 1992a), production of acute phase proteins (Ndungu, Eckersall and Jennings, 1991), and the cachexia and hypertriglyceridemia (Rouzer and Cerami, 1980; Beutler and Cerami, 1988; Ndungu, 1990). Indeed, cytokine production by the astrocytes is speculated to be involved in the
initiation of CNS pathology of HAT (Hunter et al, 1992a). On the other hand, production of certain cytokines from lymphocytes, such as IL-2 and their receptors, are down regulated during infection (Alcina and Fresno, 1985; Sileghem and Flynn, 1992). This leads to the inhibition of T-cell proliferation and to a reduced response to antigens and mitogens both of which require the secretion of IL-2 and generation of surface IL-2 receptors (Smith, 1984). This is thought to be a contributory factor to the immunosuppression and reduction of cell mitogen response observed during trypanosomiasis (Sztein and Kierszenbaum, 1993). Similarly a TLTF released from trypanosomes has been shown to induce production of interferon-γ, which subsequently stimulated parasite growth (Olsson et al, 1991). This cytokine is known to increase in vivo during trypanosome infection (Bancroft et al, 1983) and is immunosuppressive (Klimpel, Annable, Cleveland, Jerrels and Patterson, 1990). Interferon-γ may therefore be responsible for the immunosuppression that occurs during trypanosome infection (Bancroft et al, 1983; Olsson, Bakhiet and Kristensson, 1992; Bentivoglio, Grassi-Zucconi, Olsson and Kristensson, 1994).

Cytokines have also been shown to have an effect on the endocrine system. In this regard, in vivo and in vitro studies have indicated that, IL-1β and TNF-α can inhibit thyroid-stimulating hormone (TSH) secretion, as well as thyroid hormone release (Dubuis, Dayer, Siegrit-Kaiser and Burger, 1988; Van der Poll, Romijn, Wiersinga and Sauerwein, 1990; Ozawa, Sato, Han, Kawakami, Tsushima and Shizume, 1988). Similar hormonal changes have been observed during trypanosome infection (Boersma et al, 1989; Mutayoba, Gombe, Waindi and Kaaye, 1988a; Mutayoba, Oharar-Ireri and Gombe, 1988b), possibly resulting from cytokine effects on the endocrine system (Mutayoba, 1993).
1.5.2. Pathophysiological Effects of Endotoxin and the Possible Role in Pathogenesis of Trypanosomiasis

Endotoxins are components of the cell wall of gram negative bacteria that have profound effects on the immune and non immune system of many hosts (Hewett and Roth, 1993). Bacterial endotoxins are composed of complex lipopolysaccharides (LPS) with some diversity in the nature of polysaccharide but with a common lipid moiety, known as lipid-A (Fig. 1.1). Endotoxin-like molecules have also been described in a few protozoan pathogens, including, \textit{Plasmodia} species (Jakobsen, Baek and Jepsen, 1988), \textit{Trypanosoma cruzi} (Goldberge, Cordeiro Pereira and Mares-Guia, 1983) and \textit{Chlamydia} species (Lewis, Thacker and Mitchell, 1979).

In most species, the liver is the main organ for the detoxification of LPS by an intracellular element of hepatic cells (Trapani, Waravdekar, Landy and Shear, 1962), although in some species, like sheep, the lung plays a major role (Warner, DeCamp, Molina and Brain, 1988; DeCamp, Warner, Molina and Brain, 1992), while the kidney can contribute to a lesser degree (Freudenberg, Kleine and Galanos, 1984).

The \textit{in vivo} response to LPS is extremely complex, and dependent on a large number of variables, including, host species and age, dosage, timing and method of administration of LPS, and the immunological and health status of the animal (Durham, Brouwer, Barelds, Horan and Knoock, 1990; Elliot, Welty and Kuo, 1991). LPS exerts profound effects on the preformed cellular elements and has potent effect on both the cellular and humoral limb of the immune system (Bayston and Cohen, 1990). They are potent immunostimulators, strongly activating B-lymphocytes, granulocytes and mononuclear cells. In addition, LPS strongly up regulates macrophage class II major histocompatibility complex expression (Ziegler, Staffileno and Wentworth, 1984). Bacterial LPS has been demonstrated to have beneficial activities such
as immunoadjuvanticity, anti-tumour activities, and enhancement of non-specific host defence mechanisms against microbial infections (Chedid and LeGarrec, 1980; Nowotny, Abdelnoor, Behling, Butler, Johnson and Nowotny, 1982; Parat, 1983; Nowotny, 1985).

However, when LPS is administered at a high concentration or released in large amounts as may happen during an infection, it can cause adverse effects by its direct action and through the production of pro-inflammatory molecules including cytokines and prostaglandin from the mononuclear cells (Morrison and Ryan, 1979).

Bacterial LPS is endowed with a broad spectrum of biological activity such as pyrogenicity and lethal toxicity. It is the dose of LPS and duration of exposure to it that determines the effects seen in the host. Different species differ in their response to the LPS. Man is the most susceptible to LPS (Morgan, 1965; Rubenstein, Mulholland, Jeffery and Wolff, 1965; Sauter and Wolfensberger, 1980), followed by the dog (Reddin, Starzecki and Spink, 1966), while the lizard and the bird show the highest tolerance (Zuckerman and Yoeli, 1945; Goodwin and Stapleton, 1952). This could be dependent on the ability of their mononuclear cells to produce cytokines on stimulation by the LPS. Indeed, mice naturally resistant to endotoxin, are known to be unable to produce TNF (Beutler, Krochin, Milsark, Luedke and Cerami, 1986), and tolerance to endotoxin has been shown to be associated with reduced cytokine production (He, Fong, Marano, Gershenwald, Yurt, Moldawer and Lowry, 1992: Roth, McClellan, Kluger and Zeisberger, 1994).

The clinical effects of LPS are wide ranging and include fever, hypotension, hypoglycaemia, coagulopathy, organ failure, shock, and death. Although endotoxin may cause direct damage to tissue, such as, endothelium, many of its effects are produced secondary to the triggering of an inflammatory reaction which includes activation of complement, neutrophil and platelets and
release of arachidonic acid metabolites. Further secondary effects of LPS are caused by the production of cytokine in mononuclear cells (Rietschel, Brade, Schade, Seydel, Zahringer, Brandenburg, Helander, Holst, Kondo, Kuhn, Lindner, Rohrscheidt, Russa, Labischinski, Naumann and Brade, 1990; van Deventer, Buller, ten Cate, Aarden, Hack and Sturk, 1990). In this process LPS binds to a plasma protein referred to as lipopolysaccharide binding protein, forming a complex which activates mononuclear cells by attaching to CD14 surface receptor (Gally, Carrel, Glauser, Barras, Ulevitch, Tobias, Baumgartner and Heumann, 1993).

One of the initial pathological events associated with endotoxin exposure is the direct breakdown of vascular endothelium (Harlan, Harber, Striker and Weaver, 1983) including in the CNS (Eckman, King and Brunson, 1958), resulting in impairment of the endothelial barrier, diffuse increased capillary permeability, and adherence of neutrophils to the endothelium with subsequent migration into the tissue (Kopaniak, Issekutz and Movat, 1980). It also acts as a pyrogen by a direct effect on the thermoregulatory centre in the brain or indirectly via the production of IL-1 (Roth et al, 1994).

On the non-haemopoietic system, circulating LPS produces prolonged neutropenia, fever, shock, thrombocytopenia, alteration in the concentration of plasma proteins (acute phase proteins), disseminated intravascular coagulopathy (DIC), activation of the classical complement pathway and hypotension (Hewett and Roth, 1993). As mentioned, many of these effects are mediated through mononuclear cell by their release of cytokines such as TNF-α, IL-1 and IL-6. In the adipose tissue, TNF-α causes suppression of the enzyme lipoprotein lipase, thereby preventing the uptake of exogenous triglycerides by fat cells and causing the paradoxical hyperlipidaemia associated with infection (Grunfeld and Feingold, 1991) or LPS administration (Kaufmann, Matson and Biesel, 1976; Feingold, Staprans, Memon, Moser, Shigenaga, Doerrler, Dinarello and
Grudfeld, 1992; Liao and Floren, 1993). The cytokines also influence the liver function by increasing the production of positive acute phase proteins such as haptoglobin, C-reactive protein (CRP) and serum amyloid P-component (SAP), while reducing the production of the negative acute phase proteins such as albumin (Heinrich, Castell and Andus, 1990; Steel and Whitehead, 1994).

Production of prostaglandins (Peters, Karck and Decker, 1990) and nitric oxide (Rees, Cellek, Palmer and Moncad, 1990) from mononuclear cells, are also stimulated by LPS. These are known to have immunosuppressive effect on the host, while chronic exposure to endotoxin results in polyclonal B-cell activation with hypergammaglobulinaemia, profound immunosuppression and decreased mitogenic response and cytokines production (Fraker, Stovroff, Merino and Norton, 1988; Friedman, Newton, Widen, Klein and Spitzer, 1992; Roth et al, 1994).

Lipopolysaccharide leads to impairment of glucose homeostasis (Knowles, Beevers and Pogson, 1986). Early or mild endotoxaemia generates hyperglycaemia, whereas prolonged endotoxaemia causes hypoglycaemia (Filkins, 1982; Knowles et al, 1986), an effect resulting from endotoxin stimulating the production of prostaglandin (PG2α) (Kuiper, Kamps and van Berkel, 1990).

Lipopolysaccharide exposure results in the development of circulatory shock due to decreased cardiac output accompanied by a marked decrease in mean arterial blood pressure (Hinshaw, 1979; D'Orio, Wahlen, Rodriguez, Fossion, Juchmes, Haleux and Marcelle, 1987; Wakabayashi, Hatake, Kakishita and Nagai, 1987). Similarly LPS directly activates the coagulation system resulting in DIC (Warr, Rao and Rapaport, 1990), and thus a reduction in circulating platelets, clotting factors and fibrinogen (Emerson, Fournel, Leach and Redeus, 1987; Sandset, War-Cramer, Maki and Rapaport, 1991). This leads
to the formation of fibrin clots in the vasculature and ultimate deposition in tissues resulting in tissue damage due to a disruption in tissue perfusion.

Molecules with endotoxin-like activity, have been described in several parasites (see earlier) but only in a few of these has the endotoxin been compared to bacterial LPS and these include *T. cruzi* (Goldberge et al, 1983), and *Coxiella burneti* (Baca and Paretsky, 1974).

In trypanosomiasis increased endotoxin-like activity has been demonstrated in mouse serum infected with *T. b. brucei* and directly in parasite lysates (*T. b. brucei*) (Alafiatayo et al, 1993). The nature or/and chemical constituents of this endotoxin-like molecule(s), have not been characterised although it has been postulated to cause the production of pro-inflammatory molecules, including, cytokines, leading to the initiation of the pathological changes observed during trypanosomiasis (Pentreath, 1994). Bakhiet *et al* (1993) have managed to isolate and purify a TLTF molecule from *T. b. brucei* that was able to stimulate the production of interferon-γ and parasite growth. Although not fully characterised the molecule has been speculated to be a glycoprotein (Bakhiet *et al*, 1993) but it is not known whether the TLTF also has the endotoxin-like activities identified with *T. b. brucei*.

1.5.3. Acute Phase Response

The acute phase reaction is a non-specific generalised reaction of an organism following inflammation, infection, and tissue damage (Fig. 1.2) (Dinarello, 1984; Dowton and Colten, 1988; Whicher and Westacott, 1992). It is accompanied by fever, leukocytosis, concentration changes in blood hormones, iron and zinc, and in blood plasma proteins, the acute phase proteins, such as haptoglobin, C-reactive protein, serum amyloid-A and serum amyloid-P (Dinarello, 1984). The acute phase response is evoked by pro-inflammatory molecules, including, cytokines released from leukocytes, primarily monocytes,
and from damaged tissues cells, by prostaglandins and by other inflammatory mediators (Beutler, 1988; Baumann, Prowse, Marindo-Vic, Won and Jahreis, 1989; Heinrich et al, 1990). Acute phase protein synthesis and liberation is as a result of cytokine-receptor regulated hepatocyte gene expression (Perlmuter, Dinarello, Punsal and Colten, 1986).

The magnitude and type of acute phase protein response is dependent on the host and the stimulating factor. Bacterial infection and infection where tissue invasion is involved are amongst the most potent stimulators (Whicher and Dieppe, 1985). The acute phase proteins are increasingly becoming of clinical significance in both human and animal disease conditions. They are being used for the diagnosis of subclinical disease (Gruys, van Ederen, Alsemgeest, Kalsbeek and Wensing, 1993), monitoring disease activity and tissue damage (Mozes, Friedman and Shainkin-Kestenbaum, 1989), and in assessing the response to therapy (Kaneti, Winikoff, Zimlichman and Shainkin-Kestenbaum, 1984). In this respect, serum amyloid-A (SAA) has been shown to be a sensitive marker for tissue damage and disease activity, and thus a good prognostic indicator following treatment (Kaneti et al, 1984; Biran, Friedman, Neumann, Pras and Shainkin-Kestenbaum, 1986; Mozes et al, 1989). C-reactive protein (CRP), on the other hand, has been documented to be a diagnostic indicator of secondary bacterial infection and response to antibiotic treatment (Mackie, Crockson and Stuart, 1979; Schofield, Voulgari, Gozzard, Leyland, Beching and Stuart, 1982), and as a guide to differentiating bacterial from non-bacterial infections (McCarthy, Frank, Ablow, Masters and Dolan, 1978; De Beer, Kirsten, Gie, Beyers and Strachan, 1984). Haptoglobin has been documented as a good indicator of infection and inflammation (Solter, Hoffmann, Hungerford, Siegel, Denis and Dorner, 1991; Gruys et al, 1993; Skinner and Roberts, 1994), while serum amyloid-P (SAP) levels in mice with
autoimmune arthritis, were shown to correlate significantly with the severity of polyarthritis (Rodorf-Adams, Serban Pataki and Gruninger, 1985).

The acute phase proteins could also play a part in the pathogenesis of diseases in which they are produced. Haptoglobin has been shown to affect the immune system, including, mediating non-specific immune suppression (Oh, Kim and Walker, 1990), while CRP and SAP can stimulate the production of pro-inflammatory molecules from monocytes (Sarlo and Mortensen, 1985; Ballou and Lozanski, 1992). α2-Macroglobulin can modulate the immune response and inhibit mitogen induced lymphocyte proliferation (Gravagna, Gianazza, Arnaud, Neels and Ades, 1982; Miyanaga, Okubo, Kudo, Ikuta and Hirata, 1982), and also binds to cytokines thus modifying their properties (Matsudo, Hirano, Nagasawa and Kishimoto, 1989; Keith, 1990). Indeed, haptoglobin has been shown to be produced in higher amounts during trypanosome infection in resistant compared to susceptible mice (Shapiro and Black, 1992), while α-macroglobulin levels in *T. cruzi* infection were found to correlate well with resistance to acute infection (Araujo-Jorge, Lage, Rivera, Carlier and van Leuven, 1992). CRP injected into mice provided protection against *Streptococcus pneumoniae* (Mold, Nakayama, Hlzer, Gewurz and Duclos, 1981), while SAP enhanced macrophage listericidal activity (Singh, Gervais, Skamene and Mortensen, 1986).

An acute phase response detected by an increase in the serum concentration of acute phase proteins, has been documented following several parasitic infection in mice including *T. cruzi* (Scharfstein, Barcinski and Leon, 1982; Araujo-Jorge *et al*, 1992; Luz and Araujo-Jorge, 1994), *T. b. brucei* (Pluschke, Jenni, van Alphen and Lefkovits, 1986; Shapiro and Black, 1992), *Schistosoma mansoni* (Pepys, Baltz, Gomer, Davies and Doenhoff, 1979), and *Nippostrongylus brasiliensis* (LaMontagne, Gauldie, Befus, McAdam, Baltz and Pepys, 1984).
1.6. DISEASE IN THE CHRONIC MOUSE MODEL

1.6.1. Introduction

The trypanosome mouse model has been of extreme importance in the study on African trypanosomiasis (Jennings and Gray, 1983). It has been used for both pathogenesis and chemotherapy studies (Jennings, 1991; 1992a; 1992b; Hunter et al, 1992a).

Infected mice show pathological changes similar to those observed in infected humans. The mice become immunosuppressed to trypanosome antigens (Goodwin, 1970; Sacks and Askonas, 1974) and a variety of non trypanosomal antigens (Murray, Jennings, Murray and Urquhart 1974a; 1974b; Albright, Albright and Dusanic, 1977). Associated with this is a marked response of the lymphoid organs of the infected mice involving expansion of the mononuclear phagocytic system (Murray, Murray, Jennings, Fisher and Urquhart, 1974a; Morrison, Murray and Hinson, 1982) and increased levels of IgM immunoglobulins (Hudson et al, 1976). Changes in the production of pro-inflammatory molecules including prostaglandins (Fierer, Salmon and Askonas, 1984), and cytokines have also been observed in this model (Bancroft et al, 1983; Hunter et al, 1991).

1.6.2. Clinical Signs

Mice infected with trypanosomes show minimal observable clinical changes. They remain lively, but lose body condition and become anaemic, with abdominal distension associated with spleen and liver enlargement. A day or so before death, they become dull and lethargic ultimately dying of severe anaemia. Occasional posterior paresis (Jennings, Murray, Murray and Urquhart, 1974) and oedema of the eye lids are observed. In experimental models utilising deer mice, (*Peromyscus maniculatus*), advanced infections show a clinical
picture of neurological damage, lethargy, incoordination, hyperkinesis, paralysis, convulsions and coma (Molton and Stevens, 1977; Murray and Jennings, 1983).

1.6.3. Pathology

The most common pathological changes observed in infected mice include lymphadenopathy, splenomegaly, hepatomegaly with mottling and fatty change. Chronic infections are accompanied by a decrease and gelatinisation of body fat and hyperaemic brain meninges.

The lymph nodes show an increase in proliferative activity in the follicular areas. These regions become expanded due to the presence of large active germinal centres containing large lymphocytes showing high mitotic activity and a narrow layer of small lymphocytes at the periphery of the follicle. The medullary cord also contain numerous plasma cells and large lymphocytes (Morrison, et al, 1982). The thymus shows similar changes to the lymph nodes except that later in the infection there is a reduction in the thymic cortex, although there is still marked mitotic activity in the thymic cortical thymocytes. The thymic vessels become distended and sometimes contain numerous macrophages (Morrison, Murray and Bovell, 1981c). On the other hand, the spleen shows an initial proliferative phase characterised by widespread hyperplasia of the white pulp with the production of large numbers of plasma cells and an expansion of the erythropoietic component of the red pulp. Following the initial proliferative phase, a more protracted phase of further cellular proliferation leads to gradual disorganisation of the white pulp with eventual lymphoid depletion. This is also accompanied by a progressive expansion of the red pulp due to increased numbers of erythropoietic cells and to a lesser extent granulopoietic cells and macrophages (Morrison et al, 1981c).

Livers of infected mice show an increase in number and size of the Kupffer cells lining the hepatic sinusoids, many of which are found in mitosis
(Murray et al, 1974a). There is also a marked increase in cellular infiltration particularly at the perivascular locations. In addition, small foci of hepatic cellular degeneration can be found in the substance of the liver and necrotic cells are sometimes observed within the sinusoid (Morrison et al, 1982).

Mice develop a pancarditis similar to the one observed in man. This is associated with severe cellular infiltration of the endocardium, myocardium and epicardium by mononuclear cell. Also encountered is destruction of muscles fibers (Poltera et al, 1980b). Parasites are predominantly observed on the endocardial and epicardial side but are also present in the valves, the conducting system and lymphatic system draining the heart and become particularly evident in late infection. Similarly during this late infection draining lymph nodes show marked histiocytic proliferation and the cardiac blood vessels become convoluted and distended (Poltera et al, 1980b).

In the early stages of infection the brain of infected mice present a non-specific meningitis (Fig. 1.3, Fig. 1.4, Fig. 1.5). This later develops into a meningoencephalitis, with perivascular cuffing, and occasional damage to piaglial lining (Jennings and Gray, 1983). Dramatic astrocyte activation occurs (Hunter et al, 1992a). Treatment of infected mice with a number of trypanocidal drugs that do not cross the blood brain barrier and thus are unable to clear trypanosomes from the CNS, leads to the development of a severe post reactive encephalopathy (Jennings and Gray, 1983) similar to that observed in humans after treatment with melarsoprol (Adams et al, 1986). This reaction in mice has been utilised to study the pathogenesis of the disease and the associated encephalopathy. It has been shown that astrocyte activation occurs before any inflammatory changes can be observed, correlates well with cytokine production (Hunter et al, 1991; Hunter et al, 1992a) and therefore may be the core inductive event, i.e., trypanosome or trypanosome-derived factors may
activate astrocytes with the consequent development of the inflammatory reaction.

1.7. JUSTIFICATION AND OBJECTIVES OF THE STUDY

Although the clinical and pathological changes following human and animal African trypanosomiasis are well described, the primary or pathogenic mechanisms involved, and the parasite interaction with the host still remains unclear (Pentreath, 1991). Most of the pathological changes are speculated to be produced as a result of the immune response to the parasite and increased cytokine production during infection (Rouzer and Cerami, 1980; Bancroft et al, 1983; Hunter et al, 1992a). Trypanosome product(s) in vivo and in vitro, have been shown to produce some of the pathological effects observed in the infected host, including immunosuppression (Clayton et al, 1979) and stimulation of cytokines (Oka, Nagasawa, Ito and Himeno, 1989; Gichuki, 1994).

Thus, it has been hypothesised that a trypanosome product(s), possibly an endotoxin-like molecule(s), might be involved, but this has not been isolated or characterised (Greenwood, 1974; Tizard et al, 1978). Previous experimental work has demonstrated increased endotoxin-like activity in mice infected with T. b. brucei and in isolated trypanosomes (Alafiatayo et al, 1993). However, these studies did not clarify whether the endotoxin activity being measured was from the trypanosomes or from secondary bacterial infection (Alafiatayo et al, 1993). Similarly, the pathogenic significance of secondary bacterial infection or of increased serum endotoxin-like activity in the disease process in the infected host was not examined.

A trypanosome lymphocyte triggering factor (TLTF), recently isolated from trypanosomes, has been shown to stimulate lymphocytes to produce interferon gamma (IFN-\(\gamma\)) (Olsson et al, 1991). These findings seem to indicate the presence of active molecule(s) in trypanosomes which could be responsible
for the pathogenesis of trypanosomiasis. This study addresses this possibility by measuring endotoxin-like activity and the associated acute phase response in plasma of trypanosome infected animals, determining the pathological significance of such endotoxin-like activity and identifying the origin, i.e., parasitic or bacterial.

1.7.1. Objectives of the Study

1). To develop and validate an assay for serum amyloid P-component (SAP), an acute phase reactant in mice.
2). To characterise the acute phase response during chronic trypanosome infections with tissue invasive and non-invasive trypanosome species, and establish whether acute phase proteins are indicators of parasite activity or reflect tissue damage during infection.
3). To exclude secondary bacterial infection as the source of the increased serum endotoxin levels activity during murine trypanosome infection by determination of endotoxin activity in infected animals compared to infected animals receiving antibiotic treatment.
4). To determine whether the endotoxin-like molecule(s) from trypanosomes is chemically related to bacterial LPS and in particular to lipid-A, the active moiety of LPS.
Table 1.1. The classification of economically important trypanosomes.

ORDER Kinetoplastida.
FAMILY Trypanomastida.
SECTION Salivaria.

<table>
<thead>
<tr>
<th>SUBGENERA</th>
<th>SPECIES</th>
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<tr>
<td>Trypanozoon</td>
<td><em>Trypanosoma brucei brucei</em></td>
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<tr>
<td></td>
<td><em>Trypanosoma brucei rhodesiense</em></td>
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<td><em>Trypanosoma brucei gambiense</em></td>
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<td><em>Trypanosoma evansi</em></td>
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<td></td>
<td><em>Trypanosoma equiperdum</em></td>
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<tr>
<td>Dutonella</td>
<td><em>Trypanosoma vivax</em></td>
</tr>
<tr>
<td>Nannomonas</td>
<td><em>Trypanosoma congolense</em></td>
</tr>
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<td></td>
<td><em>Trypanosoma simiae</em></td>
</tr>
<tr>
<td>Pycnomonas</td>
<td><em>Trypanosoma suis</em></td>
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Fig. 1.1. A schematic representation of the structure of a gram negative lipopolysaccharide (LPS) molecule. GA: glucosamine, N: nitrogen, N-FA: N-linked fatty acids, O-FA: O-linked fatty acids, O: oxygen, $^{2}$O$_4$P: phosphate groups.
Fig. 1.2. Diagrammatic representation of the acute phase response
**Fig. 1.3.** The cerebellum of a normal mouse. Haematoxylin and eosin. x50.

**Fig. 1.4.** Brain of *Trypanosoma brucei brucei* infected mouse in late stage infection showing large number of trypanosomes in the choroid plexus of the lateral ventricle (A) with slight cellular infiltration (C). Haematoxylin and eosin x100.
Fig. 1.3

Fig. 1.4
Fig. 1.5. a and b. The cerebral section from a mouse killed in advanced stage of infection with *Trypanosoma brucei brucei* showing cellular infiltration of the meninges (C) and the perivascular spaces (D) by inflammatory cells and the presence of a Mott cell (M). Haematoxylin and eosin. x50 and x100.
CHAPTER 2
GENERAL MATERIALS AND METHODS
2.1. EXPERIMENTAL ANIMALS

2.1.1. Mice

Adult female mice used in these studies were either CD-1 or inbred NIH mice obtained from Charles River Ltd., England. They weighed between 28-35 gm body weight (bwt) and were housed in groups of six in standard mouse metallic cages. They were given food pellets and water *ad libitum*, and kept under standardised temperature, humidity and lighting condition unless otherwise stated. The animals were allowed to acclimatise to the animal house facilities for at least three weeks before being used for experimental purposes.

2.1.2. Rats

Rats used for the separation of trypanosomes, were adult white Sprague Dawley males of between 500-600 gm body weight. They were obtained from Harlan Olac Ltd., England.

2.2. TRYPANOSOME STABILATES

2.2.1. *Trypanosoma brucei brucei*-GVR 35

This was a cloned *T. brucei brucei* GVR 35/C1.6 stabilate originally isolated from a wildebeest in the Serengeti in 1966 (Serengeti /66/SVRP/10). The stabilate produces a chronic infection in mice with a survival period of at least 30 days. The parasites are visible within the circulation 4-5 days after infection (DAI), progressing into the central nervous system (CNS) and by day 21 post infection. There are considerable numbers of trypanosomes in the CNS (Jennings and Gray, 1983).

2.2.2. *Trypanosoma brucei brucei*-TREU-226

This stabilate was obtained from the Centre for Tropical Veterinary Medicine, University of Edinburgh. It produces an acute infection in rats causing fatalities within 5-10 DAI.
2.2.3. *Trypanosoma congolense*

The stabilate was obtained from the Department of Zoology, University of Glasgow. This stabilate causes a chronic infection in mice with a survival period of 30-35 days post infection.

2.3. **ANIMAL INOCULATION**

The inoculum used for infections were prepared by diluting the frozen stabilate in phosphate buffered saline (PBS), (pH 8.0), containing 1.5% glucose to give the appropriate dose in a volume of 0.2 ml per mouse. All infections were performed by intraperitoneal injection (ip), using a 25 gauge needle and 1 ml syringe.

2.4. **PARASITOLOGICAL TECHNIQUES**

2.4.1. **Monitoring Parasitaemia**

The parasitaemia in infected animals was monitored by wet smears from the tail bleeds. These were examined under the microscope at x 400 magnification. At least 20 fields were examined before an animal was recorded as being negative.

2.5. **SAMPLE COLLECTION**

2.5.1. **Blood**

Blood was removed from the tail and used for parasite examination, measurement of packed red cell volume (PCV) and serial plasma collection. At the time of sacrifice, the mice were terminally anaesthetised with carbon dioxide and bled by intracardial puncture using a 25 gauge needle and 2 ml syringe.
2.6. TISSUE FOR HISTOPATHOLOGY

2.6.1. Tissue Fixation

Several organs including the brain, liver and spleen were carefully collected from experimental animals at different stages of infection. These tissues were fixed immediately in 10% (v/v) buffered isotonic formol saline (100 ml-formalin, 4 g-acid sodium phosphate monohydrate-NaH₂PO₄·H₂O, 6.5 g-anhydrous disodium phosphate; Na₂HPO₄; made up to 1L). All tissue processing and staining were performed by the staff at the Department of Veterinary Pathology. After fixing for at least two weeks, the tissues were trimmed to the appropriate size and plane of section. The brain was cut into three sections through the fore, mid and hind brain. These were later processed for routine histopathology by the paraffin wax embedding procedure and staining with haematoxylin and eosin.

2.6.2. Tissue Processing and Staining

The fixed tissues were embedded in wax after dehydration with methylated spirit and absolute alcohol. All stages were performed in a programmable machine under vacuum and pressure. This involved putting the tissues through three, 1 hour (hr) cycles in methanol spirit, followed by three, 1 hr cycles in absolute ethanol at 40° C. This was followed by 1 hour clearing in xylene/ethanol (1:1) and another 1 hr cycle in xylene at 40° C. The tissues were then put through four cycles of wax processing at 60° C, the first two of 1 hr each followed by two, 2 hr cycles.

Sections of 3-4 µm thickness were cut and stained by haematoxylin and eosin (H/E).
2.7. HAEMATOLOGICAL TECHNIQUES

2.7.1. Estimation of Packed Red Cell Volume

The packed red cell volume (PCV) was estimated by the microcapillary method. Heparinised capillary tubes were filled to two thirds of their length with blood from the tail by capillary flow. One end was sealed with plasticine and the tubes spun in a Hawksley microhaematocrit centrifuge for 5 min. The tubes were read on a microhaematocrit reader, and recorded to the nearest percentage point.

After the estimation of the PCV, the microcapillary tubes were cut using a diamond pencil at a point 1 mm above the buffy coat. The plasma in the tube was then expelled into Eppendorff tubes by exerting pressure from one end of the tube.

2.8. BIOCHEMICAL ASSAYS

2.8.1. Endotoxin Assay

All the apparatus used for the endotoxin experiments were either new and endotoxin free, or was rendered endotoxin free by autoclaving at 180° C for 4 hr, followed by a further 2 hr in dry heat at 100° C.

All the buffers and other fluids used were endotoxin free or were rendered endotoxin free by autoclaving.

Endotoxin activity was measured using the end point-ELISA, quantitative chromogenic Limulus Amoebocyte Lysate test (LAL test) (Coatest endotoxin kit; Quadrattech Ltd, Surrey, UK.) as specified by the manufactures. The test depends of the ability of endotoxin (lipid-A moiety) to catalyse the activation of proenzyme in the Limulus Amoebocyte Lysate (LAL). The enzyme then hydrolyses the para-nitroaniline (pNA) from the substrate, Ac-Ile-Glu-Gly-Arg-pNA.HCL (S-2423). The pNA released is measured photometrically at 405 nm after stopping the reaction with acetic acid.
The standards were prepared to encompass 0.015-0.12 endotoxin units per millilitre (EU/ml) for a low range or 0.15-1.2 EU/ml for a high range. A standard curve was included in every assay performed.

Samples and standards were diluted 1:10 with endotoxin free water and heated at 75°C for 5 min in a water bath to remove non-specific inhibition of the LAL/endotoxin reaction by serum proteases. The samples and standards were allowed to cool at room temperature and mixed vigorously for 30 sec with a vortex mixer before aliquoting. The working substrate solution S-2423 was reconstituted by mixing 1:1 (v/v) of S-2423 substrate solution and substrate buffer. The LAL was reconstituted by adding 1.4 ml of LAL reagent water per vial followed with a gentle swirl. Fifty µl of test samples and standards were pipetted, in duplicate, on to a microtiter plate, followed by incubation at 37°C for approximately 5 min. Into each well, 50 µl of reconstituted LAL reagent was added, mixed and the plates incubated for a further period as specified by the manufacturer. At the end of the incubation, 100 µl of substrate-buffer solution was added to each well, followed by a further incubation as specified by the manufacture for each batch type. The reaction was terminated by the addition of 100 µl of 20% acetic acid. Absorbance was read at 405 nm on a microtiter plate reader and the sample concentration extrapolated from a standard curve drawn by plotting the absorbance against the standard concentrations, using a personal computer running the immunosoft programme.

2.8.2. Acute Phase Protein Assay

2.8.2.1. Serum amyloid P-component assay

Plasma SAP levels were measured by a direct ELISA and quantified using commercially available murine SAP standards. The primary antibody was rabbit anti-mouse SAP IgG, while the secondary antibody was donkey anti-
rabbit IgG conjugated to horseradish peroxidase (anti-rabbit HRP). This is described in more detailed in Chapter 3.

2.8.2.2. Haptoglobin assay

Haptoglobin (Hp) was measured using the method of Makimura and Suzuki (1982) with modifications by Conner, Eckersall, Wiseman and Douglas (1988). The assay uses purified bovine haptoglobin as standard. This test is based on the ability of haptoglobin to bind to haemoglobin and retain peroxidase activity at acidic pH whereas free haemoglobin loses its peroxidase activity.

Briefly haemoglobin (methaemoglobin) was prepared according to Makimura and Suzuki (1982), and stored as a stock solution at 3 g/100 ml in normal saline. Tetra methyl benzidine (TMB), in phosphate buffer, pH 3.8, was prepared according to Conner et al (1988). A stock sample of bovine haptoglobin of known concentration provided by Dr. P.D. Eckersall, Department of Veterinary Clinical Studies, University of Glasgow was used as the standard. This was serially diluted in normal saline to produce haptoglobin concentrations covering the range 0.066-2.14 g/l.

A 20 µl aliquot of the test samples and standards was pipetted into test-tubes. Into each tube was added 100 µl of the 30 mg/100 ml (1:100 dilution of stock solution) haemoglobin solution, which was mixed and allowed to react for 10 min at room temperature. Five ml of normal saline was added to each test-tube and mixed. Twenty µl from each test-tube was then pipetted into the well of a 96 well microtiter plates, each sample being prepared in duplicate. This was followed by adding 200 µl of TMB solution into each well, mixing and incubating at 37° C for 1 hr. At the end of the incubation period, 50 µl of 1% hydrogen peroxide (H₂O₂) was added to each well, and left to react until a strong blue colour developed in the high standard. The reaction was terminated
by adding 50 μl of 2 M sulphuric acid (H₂SO₄) per well. The plates were read on a Titertek multiscan ELISA plate reader at 450 nm. The haptoglobin levels within the samples were extrapolated from the standard curve which was obtained from the standard optical readings, using a PC computer connected to the reader.

In each assay control samples containing a high and low concentration of haptoglobin were included.

2.8.3. Protein Assay

The protein content of the trypanosome lysates and protein rich membranes was estimated by the biuret method on an automated biochemical analyser (Cobas Mira, Roche Diagnostics, Welynn, UK.).

2.9. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOT

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was carried out using the Laemmli buffer system (1970). This is explained in details in Chapter 6 where it was used extensively.

This employed a 15% (w/v) sodium dodecyl sulphate separating gel and 4% (w/v) stacking gel at a constant current of 20 milli-amperes (mA) per gel. The transfer on to nitrocellulose membranes was carried out using the electrophoretic method of Towbin, Staehelin and Gordon, (1979) with slight modifications as described in individual chapters. This was done overnight with a constant current of 10 mA at 8°C using a Biorad transfer apparatus (Bio-Rad Laboratories, Hercules).

Immunostaining of the membranes was carried out using the appropriate primary antibody and secondary antibodies as described in respective chapters (Chapter 3 and 6).
2.10. STATISTICAL ANALYSIS

The data for the different parameters are presented as the mean ± standard error of the mean (mean ± SEM). The significance between group means over time and the effects of the various treatments was examined by analysis of variance. This was performed using a personal computer running the statistical analysis system package (SAS, SAS Institute, Cary, N. Carolina). Group differences were considered significant when P ≤ 0.05.
CHAPTER 3
DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT
ASSAY (ELISA) FOR THE QUANTIFICATION OF MURINE SERUM
AMYLOID P-COMPONENT (SAP)
3.1. INTRODUCTION

In mammals, the acute phase response constitutes an essential physiological process of the host's non-specific defence mechanism following tissue injury, inflammation or infection (Whicher and Westacott, 1992). During this response the liver, as described in Chapter 1, through the effect of pro-inflammatory mediators including cytokines and glucocorticoids, increases its synthesis of a number of plasma proteins referred to as acute phase proteins (APP) (Baumann and Gauldie, 1994). These APP are known to have important physiological functions in the inflammatory process including inhibition of proteases and mediator pathways, and scavenging of molecule debris following tissue damage (French, 1989).

The APP are becoming of increasing clinical importance in inflammatory and infectious conditions where they have been used as indicators of infection (Skinner and Roberts, 1994), in evaluation and monitoring the degree of tissue damage (Mozes et al, 1989; Solter et al, 1991), and following the effect of certain drug treatments including a number of immunomodulatory agents (McConkey, Crockson, Crockson and Wilkinson, 1973; Dixon, Bird, Sitton, Pickup and Wright, 1984). Studies on acute phase proteins in chronic inflammation such as rheumatoid arthritis in man (McConkey et al, 1973; Dixon et al, 1984), spontaneous and collagen-induced arthritis in mice (Glatt, Blattler, Schnebli and Feige, 1984; Rordorf-Adam et al, 1985), and during different infections (Gruys, Obwolo and Toussaint, 1994; Truyens, Angelen-barrios, Torrico, van Damme, Heremans and Carliers, 1994), have proven that they are useful markers for monitoring clinical infection and inflammation (Solter et al, 1991; Skinner and Roberts, 1994).

During trypanosomiasis, the concentration of acute phase proteins has been found to increase in the plasma of mice (Shapiro and Black, 1992), dogs (Ndungu, Eckersall and Jennings, 1991), rabbits (Cook, 1979; Thomasson,

The acute phase proteins in mice include SAP (Mortensen, Biesel, Zeleznik and Le, 1983), haptoglobin (Shapiro and Black, 1992), serum amyloid-A (SAA) (McAdam and Sipe, 1976) and α₂-macroglobulin (Tunstall, Merriman, Milne and James, 1975).

Serum amyloid P-component, a normal serum protein, has been demonstrated to respond as an acute phase protein in the mouse during different pathological conditions (Pepys et al, 1979; Scharfstein et al, 1982; Luz and Araujo-Jorge, 1994). It is a glycoprotein known to be associated with virtually all kinds of systemic amyloid deposits (Skinner, Sipe, Yood, Shirahama and Cohen, 1982). The endogenous level of SAP in normal mice and during tissue injury, is dependent on the strain and age of the mice (Mortensen et al, 1983; Griswold, Hillegass, Antell, Shatzman and Hanna, 1986).

In the mouse, SAP is one of the major acute phase proteins. In contrast in man and many other species of animals (Pepys et al, 1979; Le, Muller and Mortensen, 1982), it remains relatively unchanged during disease. In the mouse, it occurs as a 23,000-dalton alpha-glycoprotein composed of 10 identical, noncovalently linked subunits arranged as two pentraxin binding face to face (Pepys, Dash, Fletcher, Richard, Munn and Finestein, 1978; Le et al, 1982). It is a molecular homologue of C-reactive protein (CRP), the prototype APP of humans (Osmand, Friedenson, Gewurz, Painter, Hofman and Shelton, 1977). The non stimulated endogenous concentration of SAP among inbred mice strain varies over a wide range, but in all the strains, SAP functions as a typical APP (Mortensen et al, 1983). The exact function of SAP is not known but it is thought to be a precursor of the glycoprotein amyloid P-component found in amyloid deposits (Skinner et al, 1982), and may therefore cause pathological lesions under certain conditions. Reported activities which may relate to its
function include modulation of blood coagulation (Fiedel and Ku, 1986), regulation of platelet reactivity (Fiedel, Ku, Izzi and Gewurz, 1983), enhancement of bactericidal activity (Singh et al, 1986), enhancement of complement-mediated phagocytosis in monocyte/macrophage populations (Wright, Craigmyle and Silverstein, 1983), stimulation of IL-1 production by monocytes/macrophages (Sarlo and Mortensen, 1985), elastin inhibition and modulation of lymphocyte function (Li, Pereira, DeLellis and McAdams, 1984; Levo and Wollner, 1985).

The concomitant stimulation of SAP and IL-1 production by ultraviolet (UV) irradiation in vivo (Gahring, Blatz, Pepys and Daynes, 1984) and stimulation of SAP production in hepatocytes by IL-1 treatment in vitro (Le and Mortensen, 1984), or following infection (Luz and Araujo-Jorge, 1994) suggests that the measurement of SAP may have significant potential in assessing the severity of immune and non-immune mediated inflammatory tissue responses mediated by pro-inflammatory molecules such as cytokines during disease. Indeed, SAP levels in mice have been shown to correlate with the activity of macrophage-derived IL-1 containing supernatants and the activity of the SAP-inducing serum factor generated following an inflammatory stimulus (Mortensen et al, 1983). In vitro work has also demonstrated that SAP is capable of stimulating mononuclear cells to release cytokines (Sarlo and Mortensen, 1985), an effect also manifested by the human homolog CRP (Ballou and Lozanski, 1992).

These observations suggest that the measurement of SAP during pathological changes might be useful in the study of the effect of anti-inflammatory and trypanocidal drugs on the basic cellular and humoral interactions associated with inflammatory responses.

Currently, available techniques for the measurement of SAP include rocket immunoelectrophoresis (Le et al, 1982), rate nephelometry (Gertz, Sipe,
Skinner, Cohen and Kyle, 1984), competitive enzyme-linked immunoassay (Truyen et al, 1994), enzyme-linked immunoassay (Luz and Araujo-Jorge, 1994; Akiyama, Sugii and Hiroto, 1992; Serban and Rodorf-Adam, 1986), and Western blot assay (Griswold et al, 1986). These assays either require isolation and labelling of SAP, or utilise substantial quantities of standard SAP preparation and anti-SAP antibodies. Most require sophisticated equipment and expertise to perform, or express the SAP levels in arbitrary units. These observations and the high cost of commercial SAP, antibodies and equipment, makes it necessary for the development of a less expensive and easy to perform assay for SAP.

Despite the fact that SAP is recognised as being one of the principle acute phase proteins in mice, the plasma levels of this protein have not been examined during experimental African trypanosome infection. Previous studies only examined C-reactive protein (CRP) and haptoglobin in this disease (Thomason et al, 1973; Basson et al, 1977; Cook, 1979; Esievo et al, 1984; Pluschke et al, 1986; Ndungu et al, 1991; Shapiro and Black, 1992).

There is great potential for utilisation of this acute phase protein in mice both in experimental trypanosomiasis and in other disease conditions (Jennings and Gray, 1983). As a first step it is necessary to determine the kinetics of SAP during the course of a trypanosome infection. To permit such work, the development of a direct ELISA to determine SAP concentration in sera of mice was undertaken.

3.2. OBJECTIVES OF THE STUDY

1). To develop a direct ELISA assay for serum amyloid P-component, an acute phase reactant in mice.
3.3. MATERIALS AND METHODS

3.3.1. Enzyme Linked Immunosorbent Assay

Reagents and equipment

Reagents were obtained from the Sigma Chemicals Company (USA) unless otherwise stated. The microtiter plates were from Greiner (Labortechnik Ltd. Dursely Scotland), the electric shaker from Luckham (Luckham Recipro-shake, Luckham Limited, Sussex, UK) and the ELISA reader from Flow laboratories (Titertek Multiscan plus, Flow Laboratories, Helsinki, Finland). The ELISA reader was connected to a personal computer (IBM PC) running the ELISA reader programme Immunosoft (Flow Laboratories, Rickmansworth, UK.).

Primary antibody

Rabbit antiserum against murine serum amyloid P-component was purchased from Novobiochem (Calbiochem Novobiochem Corporation, Beeston Nottingham, UK).

Secondary antibody

Horseradish peroxidase-conjugated donkey anti-rabbit IgG was obtained from the Scottish Antibody Production Unit, Law Hospital (Lanarkshire, UK.).

Serum amyloid P-component standard

Commercial SAP in solution at a concentration of 80-100 μg/ml was obtained from Novobiochem (Calbiochem Novobiochem Corporation, Beeston Nottingham, UK).
Coating buffer

The coating buffer consisted of 10 mM sodium bicarbonate (NaHCO₃) and 10 mM sodium carbonate (Na₂CO₄) buffered to pH 9.6 with sodium hydroxide (NaOH).

Assay buffer (Phosphate buffered saline-Tween-20) (PBST)

Assay buffer consisted of 20 mM sodium dihydrogen phosphate (NaH₂PO₄), 20 mM di-sodium hydrogen phosphate (Na₂HPO₄), 154 mM sodium chloride (NaCl) and 0.05% (v/v) Tween-20 (Sigma) buffered to pH 7.4 using sodium hydroxide (NaOH).

Blocking buffer (Phosphate buffered saline) (PBS)

The blocking buffer was 10% (w/v), instant dried skimmed milk powder (Marvel®) in assay buffer without Tween-20 (PBS, pH 7.4). The milk powder contained 1.5% (w/w) fat and 36.4% (w/w) protein.

Substrate buffer

Substrate buffer was 10 mM sodium acetate (CH₃COONa) in distilled water adjusted to pH 5.5 with citric acid.

Peroxidase substrate

The peroxidase substrate was prepared by adding into 25 ml of the substrate buffer, 100 μl of 1% hydrogen peroxide (H₂O₂) (v/v) and 400 μl of a stock solution of 0.6% (w/v) 3,3',5,5'-tetramethylbenzidine (TMB) in dimethylsulphoxide (DMSO).
Bovine serum albumin (BSA)

The bovine serum albumin was a 1% (w/v) bovine serum albumin (Sigma Chemical Company USA.) in assay buffer without Tween-20 (PBS).

3.3.2. Preparation of SAP Standards and Samples

Commercial murine SAP was serially diluted in 1% (w/v) bovine serum albumin in assay buffer without Tween-20 to cover a concentration range of 2.5-160 µg/ml. The samples were vortexted, aliquoted and stored at -20° C. Samples with low and high concentrations of SAP were always included in each assay as positive quality control samples while at the same time a 1% (w/v) BSA solution without any SAP was used as a negative control.

3.3.3. Assay Procedure

Coating

The standards and test samples were diluted 1:100 in coating buffer (pH 9.6), mixed and allowed to stand for 5 min. The diluted samples and standards (100 µl) were pipetted in duplicate into a 96 well, flat bottom microtiter plate (Greiner) and incubated at room temperature overnight (18 hr) with continuous gentle shaking on a rotary shaker.

Blocking

At the end of the coating period, the excess samples were decanted and the non-specific binding sites blocked by adding into each well 300 µl of blocking buffer (10% (w/v) dried milk in PBS). Blocking was carried out at room temperature for 1 hr with constant gentle shaking.
Washing

The excess blocking buffer was decanted and the ELISA plates rinsed three times with 300 µl per well of PBST.

Primary antibody

Washing of the plates was followed by the addition of 100 µl per well, of a 1:4,000 dilution of primary antibody (rabbit anti-mouse SAP IgG) in assay buffer. The plates were then incubated for 90 min at room temperature. At the end of the incubation, the plates were rinsed three times with 300 µl washing buffer as above.

Secondary antibody

After decanting the washing buffer, 100 µl of a 1:2,500 dilution of secondary antibody (Horseradish peroxidase conjugated donkey anti-rabbit IgG) in assay buffer was added into each well, and the plates further incubated for 90 min at room temperature. Following the incubation the excess secondary antibody was decanted and the plates rinsed four times as above.

Substrate

The peroxidase absorbed to the wells was detected by adding 150 µl of freshly prepared peroxidase substrate to each well. The plates were covered with aluminium foil and incubated in the dark for about 30 min to allow the enzyme reaction to take place.

Assay termination

The peroxidase enzyme reaction was terminated after the development of a reasonable colour in the high standard which took up to 30 min, by the addition of 50 µl of 2 M sulphuric acid (H₂SO₄) per well.
Absorbance

The absorbance was read at 450 nm wavelength using an ELISA plate reader connected to a computer. The results were analysed using the Immunosoft programme employing a linear logarithmic transformation (lin-log) in the calculation of the SAP concentration from the absorbance readings by comparing with the readings from the standards.

3.3.4. Assay Optimisation

The assay optimisation was carried out in steps as described for serum amyloid A (SAA) ELISA (Sipe, Gonnerman, Loose, Knapschaefer, Xie and Franzblau, 1989). Each step was performed at least three times.

3.3.4.1. Titration of the antibodies (primary and secondary antibody)

Initially the approximate optimal dilution of the primary and secondary antibodies were determined by coating ELISA plates with serially diluted commercial SAP that was diluted 1:100 in coating buffer. The primary antibody ranged from 1:500 to 1:20,000 while the second antibody covered the range 1:500 to 1:10,000.

3.3.4.2. Optimisation of standards and sample dilution

Standards for the ELISA were prepared as described above. The standards were diluted either 1:50, 1:100, or 1:200 in coating buffer. 100 μl aliquots from each dilution set were applied to the ELISA plates in duplicate and the assay carried out as before.
3.3.4.3. Incubation temperatures

The effect of the incubation temperature on the SAP plate binding ability was determined by coating four plates with the standard SAP preparations. Three of the plates were left overnight at 4° C and the other three at room temperature under constant gentle agitation. The remaining steps were carried out as described before.

3.3.4.4. Optimisation of incubation time for primary antibody

The influence of the incubation time for the primary antibody on the ELISA was determined. Six plates were coated overnight at room temperature (23° C) as described above. After blocking with blocking buffer, and washing, the primary antibody was added at 1:4,000 and the plates incubated for different time periods. Duplicate plates were incubated at 30 min, 90 min and 120 min. All the remaining steps were carried out as before.

3.3.5. Assay Validation

3.3.5.1. Specificity of assay

The specificity of the rabbit antibody against murine SAP was determined using the double immunodiffusion and the Western blot following separation of protein by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

3.3.5.1.1. Double immunodiffusion-Ouchterlony technique

An agarose gel of 10 mm thickness was prepared by layering boiled 1% (w/v) gel powder in Tris-buffer saline, pH 7.4 on a horizontal glass plate. This was then allowed to cool and solidify at room temperature for 15 min, followed by a further step in a humid chamber at 4° C for 15-30 min.
After solidifying, six wells were cut out around a central well. Into the central well, 5 μl antiserum was added while in the surrounding wells 5 μl of antigen was placed (acute phase serum and purified SAP). The antigen and antibody were allowed to diffuse producing a visible precipitate at points of optimal antigen-antibody concentration.

The gels were stained by the Coomassie blue stain technique. The gels were stained in Coomassie blue stain (0.5% (w/v) Coomassie blue in 9:9:2 ethanol:distilled water:acetic acid mixture) for 30 min after which they were washed with destaining solution consisting of 9:9:2 ethanol:distilled water:acetic acid mixture. The gels were allowed to destain to give minimal background colour of the gels while retaining an intense protein stain.

3.3.5.1.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blot

SDS PAGE was carried out using the Laemmli buffer system (1970). This is explained in details in Chapter 6 where it was used extensively.

For the analysis of SAP, this method employed a 15% sodium dodecyl sulphate (w/v) separating gel and 4% stacking gel at a constant current of 20 milli-amperes (mA) per gel. The transfer of the protein was carried out using the method of Towbin et al., (1979).

Serum (diluted 1:20) from normal mice and trypanosome-infected mice containing high levels of SAP and haptoglobin, and Commercial SAP in 1:1 dilution with treatment buffer (4% SDS (w/v), 20% glycerol (w/v), 10% 2,3-dihydroxybutane-1-4-dithiol (dithiothrietol) (w/v) and 0.001% bromophenol blue (w/v)) were heated in a water bath at 100°C for 5 min. After the treatment procedure, 10 μl of protein was taken and loaded into each well. The gels were run in duplicate and after electrophoresis, one was stained with Coomassie blue while the other was immunostained using rabbit anti-mouse SAP antibody after
an overnight transfer of the protein onto nitrocellulose membranes at 10 mA using a Biorad transfer apparatus.

Immunostaining of the membranes was carried out using primary antibody solution at a 1:500 dilution and secondary antibody (horseradish peroxidase-conjugated donkey anti-rabbit IgG) (Scottish Antibody Production Unit, Law Hospital, Lanarkshire, UK), at 1:1,000 dilution.

3.3.6. Assay Precision

Assay precision was determined as described by Fraser (1986). Thus, the intra assay variation was tested by preparing 20 wells in duplicate using the same sample and running in the same assay. For the inter assay variation, two quality control samples with low and high SAP levels were included in the 18 different assays performed during the study period in Chapter 4.

3.3.7. The Limit of Detection

The limit of detection was calculated as the least amount of SAP that could be distinguished from the negative control. It was calculated as the SAP concentration at 2 standard deviations (SD) away from the mean of the zero standard (Fuentes-Arderiu, 1992).

3.3.8. Clinical Validation of Assay

As a final validation of the assay, plasma samples from trypanosome-infected mice at different stages of infection were assayed for the levels of SAP. These findings are reported in Chapters 4 and 5.
3.4. RESULTS

3.4.1. Assay Optimisation

3.4.1.1. Titration of primary and secondary antibody

Results from the initial titration of the antibodies, covered a dilution range of 1:500 to 1:20,000 for the primary antibody and 1:500 to 1:10,000 for the secondary antibody. The results showed that a combination of 1:4,000 for the primary antibody (Fig. 3.1) and 1:2,500 of the secondary antibody (Fig. 3.2) showed the best results combining high signal with low background, with the highest standards giving a maximum optical density of 0.64.

3.4.1.2. Optimisation of coating temperature

Coating the plates overnight at 4° C and room temperature (23° C) resulted in similar binding in the assay, but at room temperature, the background in the negative control resulted in a slightly lower optical density thus subsequent coating was performed at room temperature (Fig. 3.3).

3.4.1.3. Optimisation of standards and sample dilution

The dilution of 1:50 gave a high optical density but high background and 1:100 and 1:200 did not differ much although the 1:100 gave a slightly lower background and a high optical density for the high standard, and it was chosen for all subsequent tests (Fig. 3.4).

3.4.1.4. Optimisation of primary antibody incubation time

Incubating the primary antibody for 60 min resulted in low optical density (OD) values while for 120 min the OD was high but with high background. Thus after addition of the primary antibody, the plate were subsequently incubated for 90 min in all subsequent tests (Fig. 3.5).
3.4.2. Assay Validation

3.4.2.1. Specificity of antibody to mouse SAP

The mouse acute phase serum, i.e., from trypanosome-infected animals, and commercial SAP tested with the rabbit SAP monoclonal antibody by double immunodiffusion gave a single band of complete identity (Fig. 3.6).

Similarly, testing the rabbit antibody to mouse SAP gave a single band (23,000 radius) in Western blots in serum from mice in acute phase and commercial SAP (Fig 3.7). Staining the blots without including first antibody did not give any bands.

An SDS-PAGE gel stained with Coomassie blue of the same sample (Fig. 3.8) confirms that SAP had a molecular radius of 23,000 and was present in the serum from trypanosome-infected mice.

3.4.2.2. Repeatability of standard curve

The absorbance and the coefficient of variance (CV) for the standard curves at 450 nm of 18 assays performed over several months are presented in Figure 3.9 and Table 3.1. The CVs of standard curve varied from 6.5 to 10.3%.

These standard curves from the different assays during studies in Chapters 4 and 5 resulted consistently in essentially equivalent standard curves.

3.4.2.3. Assay precision

The intra-assay coefficient of variation for the 20 duplicate sample wells was 4.6% at 40 μg/ml SAP concentration. The inter-assay coefficient of variation of plasma sample used as controls and analysed independently in 18 different assay runs was 12.4% at 30 μg/ml and 10.6% at 80 μg/ml SAP concentrations.
3.4.2.4. The limit of detection

The limit of detection was 5 μg/ml. This was determined as the amount of SAP that could be distinguished from the zero standard (Fuentes-Arderiu, 1992), and was calculated as the zero standard mean ± 2 SD.

3.4.2.5. Clinical validation

The assay was able to measure different SAP levels in plasma samples from mice infected with trypanosomes. This showed a rapid rise to reach peak levels of 120 μg/ml 14-16 day after infection (DAI) and then a dramatic decrease to the baseline level towards the terminal stages of infection as fully described in Chapters 4 and 5.

3.5. DISCUSSION

The results in this study demonstrate that serum amyloid P-component can be measured using a direct ELISA. The method described here demonstrates the use of a simple procedure for measuring SAP, utilising readily available SAP standard, antibodies, and equipment available in most laboratories thus allowing the assay to be easily transferable. The method is relatively inexpensive and utilises small amounts of SAP and anti-SAP antibody, unlike the alternative methods such as antibody-coated ELISA (Akiyama et al, 1992) and electroimmunoassay (Laurell, 1972) which require substantial amounts of the antigen and antibodies. The small amount of sample used for the assay makes it possible to measure SAP concentration in serial samples collected from small animals such as the mouse. To further reduce the requirement for commercial SAP, serum from mice during the acute phase response can be pooled, calibrated using commercial SAP and used as standards. Indeed pooled serum as standard eliminates any possible matrix
effect which can be a problem when standard in buffer is used to quantify analyte in serum.

The assay uses a standard curve to calculate the levels of SAP which enables the analysis of many samples at the same time. Additional benefits are the quantification of serial samples, follow-up cases undergoing pathological changes, or monitoring responses following different treatments, a task not possible with other assays such as Western blot (Griwold et al, 1986) and electroimmunoassay (Laurell, 1972).

The inclusion of a standard curve for each assay allows comparison between assays and inclusion of a high and low standard of known concentration of SAP as quality control samples in each assay, confirmed the repeatability of the assay with a CV of < 15% which is acceptable for immunoassays.

Although the physiological importance of most of the acute phase proteins is not well known, there is growing recognition of the clinical importance of APP including SAP during different pathological conditions (Kent, 1992; Sheldrick, Kent and Blackmore, 1982; Eckersall and Conner, 1988; Scott, Murray and Penny, 1992; Gruys et al, 1994), and the possible significant effect of APP in the pathogenesis of disease conditions (Shapiro and Black, 1992; Mold et al, 1981). Indeed the physiological function of SAP is not well known but the protein has been demonstrated to enhance macrophage listericidal activity (Singh et al, 1986), to agglutinate complement coated erythrocytes (Hutchcraft, Gewurz, Hansen Dyck and Pepys, 1981), and to stimulate cytokine production by mononuclear cells (Sarlo and Mortensen, 1985). Clinically, SAP levels have been shown to have a significant correlation with the severity of inflammation during polyarthitis in mice (Rordof-Adam et al, 1985). In T. b. brucei-infected dogs, an increase in the plasma concentration of C-reactive protein (CRP) and haptoglobin has been demonstrated. In this
experiment, increases in the levels of CRP, the human equivalent to SAP, were shown to correlate well with the parasitaemia, and to be a good indicator of an active infection and the efficiency of chemotherapy (Ndungu et al, 1991).

The SAP assay described here allows investigation of the kinetics of SAP synthesis during the acute phase response following trypanosome and is likely to aid studies of other disease processes.

The detection of elevated SAP levels in the serum of mice following a trypanosome infection as described in detail in Chapters 4 and 5, supports the current method's experimental potential value to monitor SAP during disease conditions, and in following the effect of immunomodulators and therapeutic drugs on the acute phase response (McConkey et al, 1973; Kusher, 1982; Kaneti et al, 1984; Eckersall and Conner, 1988). This assay will allow the study of kinetic changes and possible pathogenic significance of the acute phase response during trypanosomiasis.

3.6. CONCLUSION
The SAP assay offers distinct advantages over the existing methods in that it is simple, accurate, reproducible and sparing of antigen and antibody. The direct ELISA minimises the amount of commercial SAP and antibodies used, and at the same time enables the analysis of a larger number of samples than the existing assays, making it useful in the sequential study of disease processes. The assay will facilitate the study of SAP as a pathological disease marker and as an indicator of the response of animals following treatment with trypanocidal and other therapeutic management regimes.
Fig. 3.1. Optimisation of the ELISA for murine serum amyloid P-component: The effect of the primary antibody (rabbit anti-mouse SAP) dilution at 1:2,500, 1:4,000 and 1:20,000 with secondary antibody at 1:4,000 dilution on the standard curve.

Fig. 3.2. Optimisation of the ELISA for SAP: the effect of the secondary antibody (Donkey horseradish peroxidase conjugated donkey anti-rabbit IgG) dilution at 1:1,250, 1:2,500 and 1:10,000 and primary antibody at 1:4,000 dilution on the standard curve.
**Primary Antibody**

- 1:2500
- 1:4000
- 1:20000

**SAP Concentration (ug/ml)**

**Secondary Antibody**

- 1:1250
- 1:2500
- 1:10000

**SAP Concentration (ug/ml)**
Fig. 3.3. Optimisation of the ELISA for murine serum amyloid P-component: the effect of the coating incubation temperature at 4°C and 23°C (room temperature) with primary antibody at 1:4,000 and secondary antibody a 1:2,500 dilution on the standard curve.

Fig. 3.4. Optimisation of the ELISA for murine serum amyloid P-component: the effect of sample dilution at 1:50, 1:100 and 1:200 with primary antibody at 1:4,000 and secondary antibody a 1:2,500 dilution on the standard curve.
Coating Temperature

- 4 C
- 23 C

Fig. 3.3.

SAP Concentration (ug/ml)

Sample Dilution

- 1:50
- 1:100
- 1:200

Fig. 3.4.
Fig. 3.5. Optimisation of the ELISA for murine serum amyloid P-component: the effect of the primary antibody incubation time at 30, 90 and 120 min with primary antibody at 1:4,000 and secondary antibody a 1:2,500 dilution on the SAP standard curve.
Incubation time (First Antibody)

FIG. 3.5

Optical density

SAP Concentration (ug/ml)
**Fig. 3.6.** Specificity of primary antibody on murine SAP by the double immunodiffusion-Ouchterlony technique. Wells loaded with 5 μl of rabbit anti-mouse SAP antibody (well-1), acute phase serum (well-2), commercial SAP (well-3) and saline (well-4). The bands between the acute serum and commercial SAP gave a band of complete identity (Arrow).
Fig. 3.7. Specificity of primary antibody of the murine SAP by the Western blot. Wells were loaded with 10 μl of APR serum (lane 1), commercial SAP (lane 2), saline (lane 3) and molecular weight markers (lane 4). The primary antibody (rabbit anti mouse SAP) was used at 1:5,000 and secondary antibody (Horseradish peroxidase donkey anti-rabbit IgG) at 1:1,000 dilution. Note the single band in lane 1 and lane 2 with molecular weight of about 23,000 daltons.

Fig. 3. 8. SDS polyacrylamide gel (15% separating gel and 4% stacking gel) electrophoresis stained with Coomassie blue. Wells were loaded with 10 μl of commercial serum amyloid P-component (lane 1), acute phase serum from trypanosome-infected mice (lane 2) and normal mouse serum (lane 3). Note the SAP band with molecular weight of approximately 23,000 daltons in the lanes 1 and 2.
**Fig. 3.9.** Standard curve for the quantification of mouse-SAP ELISA (mean ± SEM).
Standard Curve

FIG. 3.9

Optical density

SAP Concentration (ug/ml)
Table 3.1. The repeatability of the SAP standard curve assessed by the coefficient of variance of the absorbency at 450 nm of standards in 18 SAP ELISA assays.
<table>
<thead>
<tr>
<th>Murine SAP (µg/ml)</th>
<th>Absorbance (Mean ± SD)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.188 ± 0.155</td>
<td>10.3</td>
</tr>
<tr>
<td>5</td>
<td>0.227 ± 0.021</td>
<td>9.7</td>
</tr>
<tr>
<td>10</td>
<td>0.265 ± 0.028</td>
<td>9.4</td>
</tr>
<tr>
<td>20</td>
<td>0.307 ± 0.031</td>
<td>8.5</td>
</tr>
<tr>
<td>40</td>
<td>0.380 ± 0.034</td>
<td>7.6</td>
</tr>
<tr>
<td>80</td>
<td>0.471 ± 0.051</td>
<td>8.6</td>
</tr>
<tr>
<td>160</td>
<td>0.615 ± 0.061</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Table 3.1. The repeatability of the SAP standard curve assessed by the coefficient of variance of the absorbency at 450 nm of standards in 18 SAP ELISA assays.
CHAPTER 4

ACUTE PHASE REACTION IN *TRYPANOSOMA BRUCEI BRUCEI* (INVASIVE) AND *TRYPANOSOMA CONGOLENSE* (NON-INVASIVE) INFECTION OF MICE
4.1. INTRODUCTION

Human and animal infection with African trypanosomiasis leads to the development of parasitaemia, anaemia, acute inflammatory reactions, immunological derangements, tissue damage and ultimately death if untreated (de Raadt and Seed, 1977; Soltys and Woo, 1977). Accompanying these pathological alterations are biochemical changes in the levels of numerous plasma components among which are the acute phase proteins (APP). Many workers have demonstrated that monitoring the plasma concentration of APP is an effective means of quantifying the immediate host response to infection (Whicher and Dieppe, 1985; Kusher and Mackiewics, 1987; Horadagoda and Ekersall, 1994).

In trypanosomiasis, the APP observed to be affected include increased plasma levels of haptoglobin (Esievo et al, 1984; Pluschke et al, 1986; Ndungu et al, 1991; Shapiro and Black, 1992), C-reactive protein (Thomason et al, 1973; Basson et al, 1977; Cook, 1979; Ndungu et al, 1991), and a decrease in albumin levels (Jenkins and Robertson, 1959).

In mammals the concentration of these acute phase proteins can increase by between 2 and 100 fold in response to many different types of tissue injury, including immune and non-immune mediated inflammation, parasitic infections and neoplasms (Stadnyk and Gauldie, 1991; Whicher and Westacott, 1992; Baumann and Gauldie, 1994). The proteins involved in the acute phase response (APR), depend on both the stimulating factors and the host species. Bacterial endotoxin is one of the most active stimulators of hepatic synthesis of APP (Whicher and Dieppe, 1985). The concentration of APP in blood is a balance between the rate of production and catabolism.

The APR is initiated by a large number of diverse inflammatory mediators, including cytokines, anaphylatoxins and glucocorticoids (Baumann and Schendel, 1991; Baumann and Gauldie, 1994). The mediators are released
initially at the site of inflammation by activated mononuclear phagocytes,
lymphocytes and other differentiated cell types, and have potent local and
systemic effects. Among the factors released in response to inflammation, are
several cytokines which specifically regulate the transcription of APP, in
particular interleukin-1 (IL-1), interleukin-6 (IL-6), and tumour necrosis factor-
α (TNF-α) (Baumann and Schendel, 1991). Of these IL-1 and TNF-α can,
indirectly via the CNS and by action on the pituitary gland, stimulate the
synthesis of glucocorticoids by the adrenal gland, resulting in a co-operative
enhancement of IL-1 and TNF-α activity. Similarly these two cytokines cause
the production of IL-6, the cytokine directly responsible for the hepatic
stimulation of the APP synthesis (Heinrich et al, 1990).

Although the pathophysiological significance of the APR is not clearly
known, the measurement of APP is becoming of increasing clinical importance
in the diagnosis and assessment of disease activity (Kent, 1992; Gruys et al,
1994), and as an indicator of tissue damage (Kushner and Mackiewicz, 1987;
Mozes et al, 1989). The known functions of APP include, mediation of local
inflammatory changes, inflammatory protease inhibition, scavenger-transport
molecules, and modification of phagocytic cell function (Pepys and Baltz,
1983; French, 1989).

In experimental studies in mice increased plasma levels of SAP have
been reported in a variety of pathological states including infections with
Trypanosoma cruzi (Scharfstein et al, 1982; Luz and Araujo-Jorge, 1994),
Nippostrongylus brasiliensis (LaMontagne et al, 1984) and Schistosoma
mansoni (Pepys et al, 1979).

Haptoglobin (HP) is another APP secreted by hepatocytes as part of the
acute phase response in most species studied (Kushner and Mackiewicz, 1987)
and has also been demonstrated in response to many types of injury (Makimura
and Suzuki, 1982; Solter et al, 1991; Skinner and Roberts, 1994), including
infection with trypanosomes (Esievo et al, 1984; Ndungu et al, 1991; Shapiro and Black, 1992). Haptoglobin is involved in scavenging of haemoglobin to aid in the recycling of endogenous haem following haemolysis (French, 1989) and has also been shown to mediate non-specific immune suppression (Oh et al, 1990).

Due to the growing clinical usefulness of APP levels as a quantitative measure of the in vivo response to infection and the emerging evidence that APP could be playing an active role in the pathogenesis of diseases, it was decided to extend knowledge of the usefulness of APP in African trypanosomiasis, using the trypanosome mouse model system. This knowledge might lead to the use of the APP in assessment of the disease condition and the response to different treatments. Indeed SAA (Mozes et al, 1989) and SAP (Rordorf-Adam et al, 1985) have been demonstrated to be good markers of tissue damage in humans and mice, respectively, while CRP has been used as a guide to the efficacy of antibiotic treatment in bacterial infection (Schofield et al, 1982).

Although APP have been found in response to African trypanosomes infections (Thomason et al, 1973; Basson et al, 1977; Cook, 1979; Esievo et al, 1984; Pluschke et al, 1986; Ndungu et al, 1991; Shapiro and Black, 1992), the mechanism of their stimulation is not known. It might be due either to parasite factors directly stimulating the cytokine network or be the result of the tissue damage caused by parasite. Indeed it is known that bio-molecules, including endotoxins, stimulate APP mediators (Knyszynski and Burger, 1971), while tissue damage due to infectious and non-infectious conditions, can result in the hepatic stimulation of APP (Whicher and Westacott, 1992). Tissue-invading parasites such as T. b. burcei could cause APP production via the latter mechanism.
An important aspect of infection with tissue invasive parasites of the brucei sub-group is that treatment with trypanocidal drugs that do not cross the blood brain barrier, do not eliminate the parasite from the CNS. This leads to major inflammatory reactions in the brain associated with parasite persistence and with increased cytokine transcripts (Jennings and Gray, 1983; Hunter et al, 1991; Hunter et al, 1992a). It is not known whether this produces a systemic acute phase reaction.

Interestingly, trypanosomes have been demonstrated to contain cytokine-inducing molecules (Oka et al, 1989; Alafiatayo, Cookson and Pentreath, 1994), while plasma from infected mice and purified parasites contain endotoxin-like activity (Alafiatayo et al, 1993). These reports would suggest that trypanosomes stimulate APP production via an endotoxin-like molecule(s). However, there have been no studies to eliminate the invasion of tissue by the parasite as the causative factor for cytokine and hence APP production.

Therefore to differentiate these mechanisms of acute phase response activation and possible pathological significance in trypanosomiasis, APP (SAP and Hp) were measured in response to tissue invasive (Jennings, Whitelaw, Holmes Chizyuka and Urquhart, 1979; Morrison et al, 1981a; Jennings and Gray, 1983) and non-invasive (Abede, Shaw and Eley, 1993) trypanosome species. In addition, it is not known whether the inflammatory reaction in the CNS, caused in HAT by trypanocidal treatment and in T. b. brucei infected mice by subcurative treatment with the trypanocidal drug (Chapter 1) diminazine aceturate, is associated with a systemic APR. Therefore APP were also monitored in T. b. brucei and T. congolense infections after diminazine aceturate treatment.
4.2. OBJECTIVES

1). To characterise the APR in *T. b. brucei* infected mice by measuring the plasma concentration of the acute phase proteins, SAP and Hp.

2). To establish whether the acute phase response occurs with an infection with non-invasive *T. congolense*.

3). To establish whether the inflammation in the CNS after trypanocidal drug treatment of *T. b. brucei* infected mice causes a systemic acute phase reaction.

4.3. EXPERIMENTAL DESIGN

4.3.1. Study One

In this experiment, the APR was monitored in inbred NIH mice infected with a tissue invasive trypanosome, *T. b. brucei* as described in Chapter 2. The animals were tail bled at 2-3 day intervals and were treated on 30 DAI with diminazine aceturate (ip) (40 mg/kg bwt). Treatment with diminazine aceturate clears the parasites from the circulation and tissues other than the CNS. This produces increased pathology in the brains of treated mice (Jennings and Gray, 1983; Chapter 1).

4.3.2. Study Two

This experiment involved monitoring the acute phase response in outbred CD-1 mice infected with a non invasive trypanosome, *T. congolense*. The animals were tail bled at 2-3 day intervals and were treated on 31 DAI with diminazine aceturate (ip). Treatment with diminazine aceturate clears all parasites from mice infected with *T. congolense*.

Different strains of mice were used in study one and two due to unavailability of the NIH mice for the second experiment.
4.4. MATERIALS AND METHODS

4.4.1. Animals

Twelve female inbred NIH and 12 female outbred CD-1 mice, of 28-35 gm body weight were used in study one and two, respectively. They were maintained in metallic cages in two groups of six in each study, fed ad lib on commercial mice pellets and allowed continual access to drinking water.

4.4.2 Trypanosomes Stabilates

4.4.2.1. *Trypanosoma brucei brucei*

This is a cloned stabilate GVR 35. The stabilate has been shown to produce a chronic infection with the mice surviving for at least 30 days (Jennings and Gray, 1983; Chapter 2).

4.4.2.2. *Trypanosoma congolense*

The stabilate was obtained from the Department of Zoology at the University of Glasgow. This stabilate causes a chronic infection in mice with a survival period of 30-35 days post infection (Chapter 2).

4.4.3. Infection

Six mice in each study were infected with approximately $10^4$ trypanosomes ip in a volume of 0.2 ml phosphate buffered saline (PBS), pH 8.0 containing 1.5% glucose. The remaining six uninfected mice were used as controls.

4.4.4. Drugs

The infected mice were treated at a dose of 40 mg/kg bwt ip, with diminazine aceturate on 31 and 30 DAI, and sacrificed on 38 and 35 DAI in the *T. congolense* and *T. b. brucei* infected mice respectively.
4.4.5. Parasitological and Haematological Techniques

The parasitaemia was monitored by wet smear and the PCV estimated by the microhaematocrit centrifugation techniques as described in detail in Chapter 2. The plasma collected from the capillary tubes was stored at -20 °C until assayed for the APP, SAP and Hp.

4.4.6. Biochemical Assays

4.4.6.1. Acute phase protein assays

4.4.6.1.1. Serum amyloid P-component

The plasma concentration of SAP was estimated by the use of the direct enzyme-linked immunosorbent assay (ELISA) described in Chapter 3.

In this assay, commercial mouse SAP (Calbiochem) was used as standard and was serially diluted to cover a SAP concentration range of 2.5-160 μg/ml. The primary antibody was rabbit anti-mouse SAP IgG (Calbiochem Novobiochem) while the secondary antibody was donkey anti-rabbit IgG horseradish peroxidase conjugate (Scottish Antibody Production Unit, Law hospital, Larnark UK.).

4.4.6.1.2. Haptoglobin assay

Haptoglobin concentrations were estimated using the method by Makimura and Suzuki (1982) with modifications by Conner et al, (1988) as described in Chapter 2.

4.4.7. Histopathology

At the end of study one and two, the mice were sacrificed, the brains removed and immediately fixed in 10% buffered formalin. These were later
processed after paraffin embedding and stained with haematoxylin and eosin stain for microscopic examination as described in Chapter 2.

4.5. STATISTICAL ANALYSIS

The results are presented as the mean (± SEM). Differences between the means in the infected and uninfected controls within each study were compared by the two way analysis of variance with repeated measures (ANOVA). Differences were considered significant at P < 0.05.

4.6. RESULTS

4.6.1. Study One: Trypanosoma brucei brucei: Invasive

4.6.1.1. Parasitaemia

After infection, parasites appeared in the blood of mice 4-5 DAI. A high parasitaemia was maintained during the course of infection although fluctuations related to parasitaemic waves were apparent (Fig. 4.1). On treatment with diminazine aceturate on 30 DAI, the parasites disappeared from the circulation by 2-3 days after treatment (DAT) and were still absent at the end of the experiment on 35 DAI.

4.6.1.2. Acute phase proteins

4.6.1.2.1. Serum amyloid P-component

The mean (± SEM) SAP concentration changes in NIH mice during the T. b. brucei infection are presented in figure 4.2. The mean SAP concentrations in the uninfected animals did not change significantly during the experimental period from the values on 0 DAI (30-35 μg/ml). In the infected mice, the mean SAP concentrations started to increase by 2 DAI, and reached peak concentration of 129.3 ± 13.2 μg/ml on 13 DAI. By 17 DAI, the mean concentration had decreased but was significantly higher than the uninfected
controls. After 17 DAI the concentrations of SAP in the infected mice kept fluctuating between 41-49 μg/ml until termination of the experiment 35 DAI. The SAP concentration in the infected mice were not affected significantly after treatment with diminazine aceturate. The mean SAP concentrations in the infected animals were significantly higher than the uninfected controls (P < 0.05) throughout the infection.

4.6.1.2.2. Haptoglobin

The mean (± SEM) haptoglobin concentrations in *Trypanosoma brucei brucei* infected and control mice are presented in figure 4.3. Haptoglobin was not detectable in plasma samples collected on 0 DAI. Following infection, serum haptoglobin was detectable by 2 DAI, and the mean concentration rose to reach a peak level of 2.02 ± 0.19 g/l on 10 DAI (P < 0.001). This was followed by a slight decline to about 1.42 g/l on 13 DAI, around which the levels fluctuated until termination of the experiment. Treatment of the infected mice with diminazine aceturate on 30 DAI did not affect the haptoglobin concentrations although the mean concentrations had started to increase slightly before the drug treatment.

In the uninfected control mice, haptoglobin was not detectable in the pre-infection serum samples or at any later stage of the experimental period.

4.6.1.3. Packed red cell volume

The mean PCV values of *T. b. brucei* infected and control mice are shown in figure 4.4. The uninfected groups of animals maintained a relatively constant mean PCV of 55-58% throughout the experimental period (P > 0.05).

The infected animals maintained preinfection PCV levels until 10 DAI. This was followed by a significant decrease in the mean PCV values on 13
DAI, a decline that continued to reach the lowest value of 38.8 ± 0.9% on 35 DAI in spite of treatment with diminazine aceturate on 31 DAI.

On statistical analysis, the infected and uninfected control mean values demonstrated a significant difference in PCV during the infection (P < 0.05).

4.6.1.4. Histopathology

The brains from uninfected mice sacrificed showed no significant changes (Fig. 4.5). The brains from infected mice treated with diminazine aceturate, 30 DAI and sacrificed 5 DAT showed marked meningitis and perivascular cuffing. The cuffs included lymphocytes, plasma cells, macrophages, and occasionally Mott cells (Fig. 4.6). A similar cellular infiltration infiltrated the meninges, with the cells also being found along the fissure and the Virchow-Robin spaces around the blood vessels forming the perivascular cuffs.

4.6.2. Study Two: *Trypanosoma congolense*: Non-invasive

4.6.2.1. Parasitaemia

After infection with *T. congolense*, trypanosomes were detected by 3-4 DAI. The parasites were demonstrated in all infected animals at the sampling times (Fig. 4.8). Following treatment with diminazine aceturate on 31 DAI, the parasites could not be detected in any animals by 2-3 DAT and remained negative until the end of the experimental period 38 DAI.

All the infected mice maintained a relatively high parasitaemia with fluctuations common in trypanosome infection.
4.6.2.2. Acute phase protein

4.6.2.2.1. Serum amyloid P-component

The changes of mean plasma concentrations of SAP in the *T. congolense* infected and uninfected controls are depicted in figure 4.9. The uninfected mice had a mean SAP level of 65-75 µg/ml which did not vary significantly during the experimental period (P > 0.05). Following infection, the mean plasma SAP concentrations started to increase within 2 DAI reaching a mean peak concentration of 144.6 ± 8.5 µg/ml at 12 DAI. This was followed by a decrease to a mean of 86.8 ± 8.2 µg/ml on 21 DAI and then an increase which was more pronounced and significant, following diminazine aceturate treatment, 31 DAI, to reach a second peak of 152.6 ± 5.6 µg/ml on 34 DAI. Thereafter, the mean plasma SAP concentration started to decrease until termination of the experiment but was still significantly higher than the control concentrations.

Statistical analysis revealed that the infected animals showed a significant increase in the mean plasma SAP concentrations compared to the uninfected control animals (P < 0.05).

4.6.2.2.2. Haptoglobin

The mean haptoglobin concentrations in the infected and uninfected CD-1 mice are depicted in figure 4.10. The uninfected controls had a mean haptoglobin plasma concentration of 0.12-0.22 g/l, which did not vary significantly throughout the experimental period.

On infection, the mean plasma concentration increased significantly from 0.15 ± 0.02 g/l on 0 DAI, to reach a peak concentration of 2.86 ± 0.33 g/l 7 DAI (P < 0.001). This was followed by a decrease at 14 DAI, to about 1.3-2 g/l at which level it fluctuated until the animals were treated with diminazine aceturate, on 31 DAI. Following treatment, the haptoglobin concentration in the
infected animals decreased rapidly from 34 DAI to just above uninfected values by 36 DAI, where it remained until termination of the experiment on 38 DAI.

The mean plasma haptoglobin concentrations in mice following infection were significantly higher when compared to uninfected controls (P < 0.05).

4.6.2.3. Packed red cell volume

The mean PCV values in the uninfected controls and infected mice are depicted in figure 4.11. The uninfected controls did not show any significant changes in the PCV values which ranged from 52 to 55% throughout the experimental period.

After infection, the mean PCV values in the infected mice started decreasing around 7 DAI, reaching a low mean value of 36.8 ± 1.6% on 21 DAI. This was followed by a slight recovery thereafter, which was more marked after the diminazine aceturate treatment on 31 DAI and reached the pre-infection values of 55.7 ± 0.5% by 4 DAT where it was maintained until termination of the experiment 38 DAI.

4.6.2.4. Histopathology

There were no noticeable changes in the histopathology of brains from the infected CD-1 mice treated on 31 DAI and sacrificed 7 DAT (Fig. 4.12). Similarly the uninfected CD-1 mice did not show any detectable changes (Fig. 4.13).

4.7. DISCUSSION

Infection with T. b. brucei and T. congolense in study one and two respectively, resulted in the development of anaemia, and an acute phase response reflected by an increase in the plasma concentrations of SAP and Hp.
However, pathological changes in the brain, after diminazine aceturate treatment only occurred with *T. b. brucei* infection.

Anaemia was manifested by a decrease in the PCV values which started to decline around 8 DAI in the *T. congolense* and 10 DAI in the *T. b. brucei* infections respectively. Treatment with diminazine aceturate on 31 and 30 DAI in the *T. congolense* and *T. b. brucei* respectively, resulted in disappearance of parasites from the circulation 2-3 DAT in both infections. There followed a rapid recovery of the PCV values to pre-infection levels in *T. congolense*, whereas treatment had little effect in the PCV values in *T. b. brucei* infected animals which remained low up to the end of the experiment. This difference was considered significant and it was concluded that the experimental design with a 2 DAT longer recovery period in *T. congolense* mice had no effect on the results.

In both infections, the acute phase proteins, SAP and Hp, rose rapidly from pre-infection concentrations in CD-1 and undetectable levels for haptoglobin in NIH mice to reach peak concentrations 12 and 13 DAI for *T. congolense* and *T. b. brucei* infections, respectively. The SAP levels in both infections dropped to relatively low concentrations by about 17-20 DAI, although they were still above the uninfected control concentrations. In *T. b. brucei* infections the SAP concentrations did not change much thereafter, except for a slight increase on 25 DAI, and were not significantly affected by diminazine aceturate treatment. In contrast in *T. congolense*-infections, the SAP concentrations increased giving a second peak 34 DAI which was not significantly affected by drug treatment although the concentrations had started decreasing in the samples taken on 36 and 38 DAI.

Haptoglobin increased significantly from pre-infection and undetectable concentrations in *T. congolense* and *T. b. brucei* infected mice, respectively. The concentration of this protein reached peak concentrations on 7 and 10 DAI.
in *T. congolense* and *T. b. brucei* infections, respectively. Thereafter, in both infections, the concentrations remained elevated but in *T. congolense*, the concentrations of Hp decreased dramatically following diminazine aceturate treatment to just above control concentrations at the end of the study, whereas in *T. b. brucei*-infected animals, the Hp concentrations remained elevated by the end of the study and were not significantly affected by treatment.

The two strains of mice had different pre-infection endogenous concentrations of both SAP and Hp. SAP was demonstrable in both strains but was found to be higher in the CD-1 mice. This strain difference in SAP has also been observed to occur in other strains of mice (Pepys *et al.*, 1979; Mortensen *et al.*, 1983). Similarly the two strains of mice showed differences in the endogenous non-stimulated haptoglobin concentrations. Haptoglobin was demonstrable in CD-1 mice and was absent or below the level of detection in the NIH mice. Haptoglobin concentrations in different strains of mice have previously been shown to vary in amount and in some strains, found at low or undetectable concentrations in normal animals (Peacock, Gelderman, Ragland and Hoffman, 1967). Haptoglobin has also been found to vary with age of the mice (Palmer, 1976). It was unfortunate but unavoidable that two different strains of mice were used in these studies. Nevertheless, this did not affect the main finding that SAP and haptoglobin are acute phase reactants during murine trypanosome infections and that this occurs with both tissue-invasive and non-invasive trypanosome species.

Although haptoglobin concentrations in plasma have previously been documented to increase in response to trypanosome infections in different species including dogs (Ndungu *et al.*, 1991), mice (Shapiro and Black, 1992), and cattle (Esievo *et al.*, 1984), this is the first time that SAP has been shown to react as an acute phase protein in African trypanosomiasis. At the same time, this is the first study to quantify haptoglobin in a murine model of African
trypanosomiasis as previous studies had only demonstrated haptoglobin by qualitative and semi-quantitative methods (Shapiro and Black, 1992). Furthermore it was shown that there is acute phase proteins increase in response to both invasive and non-invasive trypanosome infections.

The haptoglobin and SAP reactions were similar in that large initial responses to infection were observed, but were dissimilar in that the haptoglobin concentration remained at an elevated concentrations while the SAP value decreased to a similar concentration as the uninfected controls. This difference in the response of the two proteins could be because haptoglobin is more responsive to long term stimulation or due to it having a longer plasma half life of 3 days (Koj, 1974), compared to 7-8.25 hrs for SAP (Baltz, Dyck and Pepys, 1985). A combination of these effects would thus make haptoglobin a better indicator of chronic infections. Indeed in cattle with different inflammatory conditions, levels of haptoglobin and serum amyloid-A (SAA) expressed as a haptoglobin/SAA ratio have been used to indicate the chronicity of the underlying condition (Alsemgeest, Kalsbek, Wensing, Koeman, van Enderen and Gruys, 1994).

Similar differences in the response of acute phase proteins have also been observed in the response of SAP and α2-macroglobulin in mice infected with *T. cruzi* (Luz and Araujo-Jorge, 1994). Similar results in the SAP response showing an initial increase to peak levels followed by a decrease, in spite of continued stimulation, has also been observed in *Schistosoma mansoni* infections in mice (Pepys *et al*, 1979), and in amyloidosis tolerant and susceptible mice injected daily with casein. In the latter experiment, SAP concentrations in the susceptible mice strains increased and remained elevated while in the tolerant strains the concentrations rose and later dropped in spite of continued stimulation (Baltz, Gomer, Davies, Evans, Klaus and Pepys, 1980).
Haptoglobin plasma concentrations have been shown to decrease or be depleted by intravascular haemolysis which has been reported in trypanosome infection in cattle infected with *T. vivax* (Esievo et al, 1984). In both the *T. b. brucei* and *T. congolense* infections, haptoglobin levels remained elevated in spite of the development of severe anaemia. This suggests that the anaemia was not caused by intravascular haemolysis which would increase the plasma clearance of haptoglobin (Koj, 1974). Similar findings were observed in dogs infected with trypanosomes (Ndungu et al, 1991).

Following infection, CD-1 mice gave higher haptoglobin responses to *T. congolense* than NIH mice gave to *T. b. brucei*. This difference in response could be due to differences in the trypanosome species, *T. b. brucei* and *T. congolense*, or the strain of mice used. However, as *T. b. brucei* is tissue invasive, this species would have been expected to result in higher values due to the tissue damage resulting from direct effect of the parasite and/or inflammatory responses to the parasite in tissue. This is in contrast to *T. congolense* which is generally restricted to the vascular system (Abede et al, 1993), although also accompanied with tissue damage (Sekoni, Njoku, Kumi-Diaku and Saror, 1990; Katunguka-Rwakishaya, 1992). In contrast to infection with *T. b. brucei* which results in severe cellular infiltration and tissue damage in most tissues, with *T. congolense* infection these changes are much less severe (Murray, 1974).

The difference due to the strains of mice used could also affect the haptoglobin response. A difference in haptoglobin response following trypanosome infection has been demonstrated in mice with known differences in their resistance to trypanosomiasis (Shapiro and Black, 1992). In their study, the more resistant strain resulted in higher haptoglobin production; a similar effect was observed in this study where CD-1 mice produced higher levels than NIH mice which are less resistant to infection (Jennings, unpublished...
observation). Similarly, the difference in mouse strains might be due to the ability of the CD-1 mice to produce higher levels of cytokines as demonstrated with TNF-α production (Haranaka, Satomi and Sakurai, 1984). Indeed in trypanosome infections in mice, the more resistant strains have been demonstrated to produce higher levels of interferon (DeGee, Sonnnefeld and Mansfield, 1985).

The observation that the CD-1 mice infected with *T. congolense* gave two peaks of SAP while the NIH mice had a small second peak might also be related to the patterns of cytokine production in the two strains of mice. Differences in cytokine concentrations and pattern of response have been observed in trypanosome infected mice. The more trypanosome resistant strains of mice such as B10.BR, gave higher second peaks of interferon, whereas, this second peak was either of small magnitude or absent in the less resistant strains (CBA) (DeGee *et al*, 1985). Therefore, this difference could be related to the different strain of mice or the parasite used. Different strains of mice have been shown to respond to the same stimulus giving different levels of SAP (Baltz *et al*, 1980; Le *et al*, 1982; Mortensen *et al*, 1983). Indeed the response of SAP in some of these mice was found to be dependent on the ability of macrophages to produce interleukin-1 (Mortensen *et al*, 1983).

Whether this differences in response of SAP and haptoglobin is due to differences in the trypanosome species or the strain in the mice used can not be clearly elucidated by this study. Further work needs to be done using the same trypanosome species on the different strains of mice.

The observation that the haptoglobin concentrations decreased rapidly following diminazine aceturate treatment in the *T. congolense* and not *T. b. brucei* infection, is likely to be due to the treatment being curative for *T. congolense* (Abede *et al*, 1993), as opposed to *T. b. brucei* infection where it is known that this treatment does not clear the parasite from the CNS. This effect
might be further aggravated in the *T. b. brucei* infection, as it has been shown that increased CNS pathology occurs after administration of diminazine aceturate in mice with established CNS trypanosome infection, a phenomenon associated with increased cytokine transcripts and thus possibly cytokine proteins in the CNS (Hunter, Gow, Kennedy Jennings and Murray, 1991; Hunter *et al*., 1992a). Intracerebroventricular injection of IL-1 and leukocytic pyrogens, have been shown to result in increased response in the hepatic synthesis of haptoglobin and CRP (Turchik and Bornstein, 1980; Dao, Bell, Feng, Jameson and Lipton, 1988) and a similar process might be in operation here. The possible effect of the CNS on the hepatic acute phase response was not studied in this experiment and further experiments are needed to identify the effect of the CNS infection on the hepatic acute phase response.

The occurrence of SAP and Hp increases during trypanosomiasis might have a useful role in this trypanosome mouse model as sensitive markers for inflammation or trypanosome activity. However, it will be important to identify the mechanisms responsible for the development of the APR. This study has shown that, in mice, an APR occurred in infections of both invasive and non-invasive species of trypanosome. It was notable that increased SAP and Hp were detected in both infections before trypanosome could be demonstrated in circulation.

Acute phase proteins might have pathophysiological functions during disease conditions such as trypanosomiasis. Haptoglobin can influence the pathogenesis of disease in several ways. In the circulation, it binds iron, an effect that in the long run could improve iron conservation which would mitigate the chronic anaemia which is a major pathologic consequence of trypanosomiasis. Also, the peroxidase activity of the haemoglobin-haptoglobin complexes, might locally inactivate inflammatory cell products which cause tissue damage in chronic disease (Koj, 1974.). Haptoglobin, at acute phase
response concentrations, has been shown to be able to significantly depress T-cell stimulation (Oh et al., 1990), a common phenomenon during trypanosomiasis (Alcina and Fresno, 1985; Sileghem and Flynn, 1992). In addition, SAP has been shown to agglutinate complement-coated antibody sensitised erythrocytes (Hutchraft et al., 1981), enhance interleukin-1 production mediated by monocyte/macrophage cells (Sarlo and Mortensen, 1985), and also enhance macrophage listericidal activity (Singh et al., 1986). Thus in trypanosomiasis high levels of SAP in circulation might augment cytokine production of the monocyte/macrophage cells, and by agglutinating red blood cells, SAP might contribute to the destruction of the erythrocytes by the reticuloendothelial system leading to the development of anaemia.

The measurement of haptoglobin has proven to be a useful pointer to the severity of infection in cattle (Skinner, Brown and Roberts, 1991) and a useful marker for bacterial infection in sheep (Skinner and Roberts, 1994), cattle (Blackshaw, 1979; Makimura and Suzuki, 1982; Conner, Eckersall, Doherty and Douglas, 1986; Eckersall, Parton, Conner, Nash, Watson and Douglas, 1988; Skinner et al., 1991) and dogs (Solter et al., 1991). In these cases, it was found to be more sensitive, specific, efficient and less likely to give false positive and negative results in comparison to other indicators such as haematology (Skinner and Roberts, 1994).

Serum amyloid-P component has been found to correlate well with the severity of polyarthritis (Rordorf-Adam et al., 1985), and CRP the human SAP homologue, has been shown to correlate well with the level of trypanosome parasitaemia (Ndungu et al., 1991). In mice, a good correlation between the ability to produce IL-1 and the response of SAP has been demonstrated (Mortensen et al., 1983). Similarly haptoglobin levels in infected mice was found to increase at higher levels in trypanosome resistant mice, compared to the susceptible strains (Shapiro and Black, 1992), and also stimulation of Hp
has been shown to be related to the ability of the inflammatory cells such as macrophages to produce the stimulating factors (Palmer, 1976). Therefore the circulating levels of APP have the potential to be used as markers for the overall activity of inflammatory mediators.

Indeed APP have advantages over other clinical indicators of inflammation and infection, such as the measurement of the levels of pro-inflammatory cytokines, haematology and enzyme activity. Assays for cytokines in biological fluids, are difficult and expensive to perform. The very transient nature of the cytokines in the circulation due to their short plasma half-life, makes it difficult to detect any peak levels (Hol, Snel, Draaijer and Gruys, 1987), and in certain diseases localisation of the cytokines occurs and they remain in the area of production thus not detectable in most biological fluids (Waage et al, 1989).

Although the measurement of the levels of individual cytokine might not give a true picture of their effect in vivo, it would be of interest to establish the levels of the peptides during a trypanosome infection. The cytokine interaction in vivo is complex and their effect can be influenced by the presence of other biologically active agents including hormones, growth factors and prostaglandins. Indeed, cytokines have been known to be synergistic (Elias and Lentz, 1990; Neta, Sayers and Oppenheim, 1992; Lee, Klampfer, Shows and Vilcek, 1993), antagonistic (Kamijo, Shapiro, Le, Huang, Anguet and Vilcek, 1993), and even in some situations produce a complete different effect than individual cytokines (Feinman, Henriksen-DeSteano, Tsujimoto and Vilcek, 1987; Gauldie, Richard, Northemann, Fey and Bauman, 1989). This is a major drawback in the interpretation of the results from the measurement of cytokines in biological fluids during disease, such as in investigations of trypanosomes infection. The levels of the APP on the other hand, remain elevated for at least 24 hours and sometimes for 2-3 days in circulation and also represent the
outcome of the interaction of the different molecules in the host and between different cytokines produced during disease.

The current approach to monitoring inflammation in infectious disease includes haematological screening. This requires a whole blood sample that is freshly drawn for analysis. In contrast, APP are not only more stable than cellular components of blood, but also the assays can be performed on previously frozen serum samples. Indeed haematological analyses in trypanosomiasis might not be useful as inflammatory indicators because of the inconsistency of the results which depend on the type of trypanosome and the host involved (Paling, Moloo, Scott, Mcodimba, Logan-Henfrey, Murray and Williams, 1991; Katunguka-Rwakishaya, 1992). Nevertheless, it must be emphasised that haematological screening is an important component of trypanosome infections which can be accompanied by anaemia and leucopenia associated with neutropenia, lymphopenia, eosinopenia and monocytosis, effects attributed to haemopoetic depression and sequestration of the cellular components in the tissue and the mononuclear phagocytic system (Anosa, 1983).

Acute phase protein assays are also less affected by transient physiological stimuli, such as excitement, although exogenous glucocorticoids can affect some assay results (Harvey and West, 1987). Acute phase protein assays can also aid in determining the existence of inflammation in animals with bone marrow suppression or depletion (Jain, 1989) as occurs in trypanosomiasis (Andrianarivo, Muiya, Oppollo and Logan-Henfrey, 1995). This situation, the bone marrow being a major haematopoetic centre and thus source of inflammatory cells, can result in a reduced blood cells and inflammatory response which can significantly affect haematological cell counts results.
The measurement of APP as indicators of activity of pro-inflammatory molecules and pathological changes during infection is likely to be important in the study of the pathogenesis of African trypanosomiasis giving an overall view of the integrated outcome of the cytokine network. During infection with trypanosomiasis, there are many changes accompanied by activation of the mononuclear system/polymorphonuclear system (Murray et al, 1974a), and an increase in the production of cytokines including TNF, IL-6, IL-1 and interferon-γ among others (Bancroft et al, 1983; Reincke et al, 1993). These pro-inflammatory molecules are postulated to be responsible for generating the inflammatory changes observed in the CNS (Pentreath et al, 1990; Hunter et al. 1991) and general circulation during trypanosomiasis (Rouzer and Cerami, 1980).

4.8. CONCLUSION

This study has demonstrated that SAP and haptoglobin are acute phase reactants during chronic murine trypanosomiasis. SAP and haptoglobin, responded immediately but SAP concentrations dropped more rapidly than haptoglobin which remained elevated throughout the infection in both T. b. brucei and T. congolense infections. The results suggest the mediation of pathological changes during murine trypanosomiasis, such as cytokine production and thus APP, starts early before the demonstration of parasites in the circulation, and is not dependent on the invasiveness of the parasite but possibly occurs through the release of active molecule(s) by the parasites. In addition, the measurement of APP during infection confirms their potential use as indicators of infection, monitoring efficacy of treatments, and in the study of the pathogenesis of trypanosomiasis.
Fig. 4.1. Changes in the parasitaemia in mice infected with *Trypanosoma brucei brucei* and treated with diminazine aceturate (B) 30 DAI.
Fig. 4.2. Mean (± SEM) plasma serum amyloid P-component concentration changes in *Trypanosoma brucei brucei*-infected and control NIH mice, and following subcurative treatment with 40 mg/kg diminazine aceturate (B) on 30 DAI.

Fig. 4.3. Mean (± SEM) plasma haptoglobin concentration changes in *Trypanosoma brucei brucei*-infected and control NIH mice and following subcurative treatment with 40 mg/kg diminazine aceturate (B) on 30 DAI.

Fig. 4.4. Mean (± SEM) packed red cell volume changes in *Trypanosoma brucei brucei*-infected and control NIH mice and following subcurative treatment with 40 mg/kg diminazine aceturate (B) on 30 DAI.
Fig. 4.2. INFECTED vs. UNINFECTED

SAP CONCENTRATION (µg/ml)

Fig. 4.3. INFECTED vs. UNINFECTED

HAPTOglobin CONCENTRATION (g/l)

Fig. 4.4. INFECTED vs. UNINFECTED

PCV LEVELS (%)
Fig. 4.5. The cerebellum of a normal NIH mouse. Haematoxylin and eosin. x100.
Fig. 4.6. Brain from a NIH mouse with advanced infection with *Trypanosoma brucei brucei*. Note the dilatation and massive trypanosomal infiltration of the lateral ventricle choroid plexus (T) and occasional lymphocyte and plasma cell (C). Haematoxylin and eosin. x100.

Fig 4.7. Cerebellum from *Trypanosoma brucei brucei* infected NIH mouse with a subcurative treatment at dose of 40 mg/kg (ip) with diminazine aceturate 30 DAI and sacrificed on 35 DAI, showing severe meningitis and perivascular cuffing. x50.
Fig. 4.8. Changes in the parasitaemia in mice infected with *Trypanosoma congolense* and treated with diminazine aceturate (B) 31 DAI.
Fig. 4.9. Mean (± SEM) plasma serum amyloid P-component concentration changes in *Trypanosoma congolense*-infected and control CD-1 mice and following curative treatment with diminazine aceturate (B) on 31 DAI.

Fig. 4.10. Mean (± SEM) plasma haptoglobin concentration changes in *Trypanosoma congolense*-infected and control CD-1 mice and following curative treatment with diminazine aceturate (B) on 31 DAI.

Fig. 4.11. Mean (± SEM) packed red cell volume changes in *Trypanosoma congolense*-infected and control CD-1 mice and following curative treatment with diminazine aceturate (B) on 31 DAI.
Fig 4.12. Cerebral cortex from *Trypanosoma congolense*-infected mouse and with a curative treatment with diminazine aceturate 31 DAI and sacrificed on 38 DAI. There were no detectable histological changes. Haematoxylin and eosin. X100.

Fig. 4.13. The cerebral cortex of a normal CD-1 mouse. Haematoxylin and eosin. x50.
CHAPTER 5

THE EFFECT OF ANTIBIOTIC TREATMENT ON THE PLASMA LEVELS OF ENDOTOXIN ACTIVITY, ACUTE PHASE PROTEINS AND THE PATHOLOGICAL CHANGES IN MICE INFECTED WITH TRYpanosoma brucei brucei
5.1. INTRODUCTION

The African trypanosome causes a myriad of changes of a physical, neurological, immunological, and biochemical nature in infected human and animal hosts, eventually leading to death (Murray, Morrisson and Whitelaw, 1982; Molyneux et al, 1984). The specific mechanisms leading to these pathological alterations are not yet fully characterised and so there has been a continuous search for the primary event(s) and factor(s) involved in initiating these changes. Various hypothesis have been proposed to explain the pathological events, including the possible production by the parasite of toxin-like molecule(s) (Tizard et al, 1978) and various forms of immune-mediated pathology in the host (Mansfield, 1990).

The existence and involvement of parasite-derived molecule(s) similar in action to bacterial endotoxin has been suggested and it may be responsible for the hypergammaglobulinaemia and polyclonal cell activation observed in trypanosomiasis (Greenwood, 1974). Recent experimental work has demonstrated an increase in the levels of such endotoxin-like activity in the plasma of trypanosome-infected mice, and purified trypanosomes demonstrated high endotoxin activity as measured by the in vitro limulus amoebocyte lysate test (LAL); the recognised method for determination of endotoxin activity (Alafiatayo et al, 1993).

The possible involvement of a trypanosome endotoxin has been further supported by the observation that trypanosome product(s), when administered to animals, have pronounced biological activity similar to endotoxin preparations. Thus trypanosome products are mitogenic and cause immunosuppression (Assoku and Tizard, 1978; Clayton et al, 1979; Albright and Albright, 1981). In addition, trypanosomes are known to produce molecules that inhibit cell growth (Sztein and Kierszenbaum, 1991; Temitope et al, 1993), and whole viable trypanosomes or non viable trypanosome lysate have been
shown in vitro to stimulate the production of cytokines in astrocytes (Gichuki, 1994), and macrophages (Hotez, Le Trang and Cerami, 1984). Furthermore, prostaglandin production has been stimulated in astrocytes and fibroblasts (Alafiatayo et al., 1994) by similar trypanosome preparations. In vivo injection of crude trypanosome membranes into mice results in increased granulocyte-macrophage colony stimulating activity in their serum (Oka et al., 1989), an effect known to be due to the cytokine, granulocyte-macrophage stimulating factor (GM-CSF). Meanwhile, in vitro investigation has shown that macrophages harvested from trypanosome-infected animals, show alteration from normal in their ability to produce cytokines and express cytokine receptors upon stimulation with several immunostimulants, including, LPS and Concanavalin-A (Sileghem, Darji, Hamers and De Baetselier, 1989).

Interactions between trypanosome and lipopolysaccharide (LPS) have been reported in vivo. The administration of bacteria or LPS, at different stages during a trypanosome infection, influences the course of the infection (Singer, Kimble and Ritts, 1964; Murray and Morrison, 1979). LPS injection before, during or shortly after infection increases resistance while if administered after an established infection, results in reduced resistance as measured by the survival rate, possibly indicating a common mechanism of action between the trypanosomes and LPS.

The presence of an endotoxin-like substance has been demonstrated in other parasites, notably Plasmodium species (Jakobsen et al., 1988), Sarcocystis cruzi (Fayer, 1988), Chlamydia (Lewis et al., 1979), and Trypanosoma cruzi the latter being closely related to the African trypanosome (Goldberg et al., 1983).

Among the pathological changes in the host which are common to trypanosome infection and to administration of LPS are immunosuppression, hypergammaglobulinaemia, polyclonal cell activation (Greenwood, 1974; Murray et al., 1974a; 1974b; Roelants, Pearson, Morrison, Mayor-Whithey and
Lundin, 1979), and the production of pro-inflammatory molecules such as cytokines (Hewett and Roth, 1993; Reincke et al, 1993). These pro-inflammatory molecules are increasingly being recognised as important mediators of the different pathological alterations observed in trypanosomiasis (Rouzer and Cerami, 1980; Pentreath et al, 1990; Hunter et al, 1991).

Bacterial LPS, and disease conditions where tissue is damaged, are among the most potent inducers of acute phase protein synthesis (Whicher and Dieppe, 1985). Indeed, SAP which is an APP in mice, has been shown to be very sensitive to endotoxin (Poole, Gordon, Baltz and Steinning, 1984), and haptoglobin has been documented to respond to bacterial infections making it a useful and sensitive indicator of bacterial infection in cattle (Blackshaw, 1979; Makimura and Suzuki, 1982; Conner et al, 1986; Eckersall et al, 1988; Skinner et al, 1991). This phenomenon has made it possible to utilise the assay of APP as markers of inflammation (Mozes et al, 1989; Kent, 1992; Kusher and Mackiewicz, 1987) and in the diagnosis of complications resulting from secondary bacterial infection occurring in other disease conditions (Mackie et al, 1979; Becker, Waldburger, Hughes and Pepys, 1980). When serial serum samples are taken during secondary bacterial infections, the measurement of the acute phase proteins is valuable in assessing the response to antibiotic therapy (Philip, 1982; Schofield et al, 1982; Walker, Rogers, Riches, White and Hobbs, 1984; Whicher and Dieppe, 1985).

Chapter 4 demonstrated that an APR occurs in mice infected with *T. b. brucei* (a tissue invasive parasite). As the response also occurs with *T. congolense* (a non tissue invasive parasite), it is possible that the APR is a direct parasite stimulated response rather than a response to tissue damage. Therefore, the APP can be used to study the likelihood that stimulation may be caused by production of trypanosome endotoxin-like activity as demonstrated in Chapter 4. Of the APP, SAP and Hp are suitable for study of infection in mice.
Previous studies have searched for the origin of the increase in plasma endotoxin-like activity, and the possible presence of such a molecule in trypanosomes. However, difficulties have been encountered in confirming the source of the activity as being from trypanosome parasites rather than from other pathogens in the host such as secondary bacterial infection or gastrointestinal tract (GIT) bacteria (Alafiatayo et al., 1993). Although there are no data available showing a significant contribution by secondary bacterial sources in the pathogenesis of HAT, in *T. cruzi* infection, bacterial LPS from the GIT has been postulated to affect the pathology of the infection (Bambirra, Cruz, Campos and Lima, 1984) and increased absorption of LPS from the GIT following intestine damage has been documented (Caty, Guice, Oldham, Remick and Kunkel, 1990).

Antibiotic treatment provides a means to investigate these areas. Previous observations (Dr. FW. Jennings, unpublished observations) have indicated that *T. b. brucei* infected mice treated with antibiotics norfloxacin and ciprofloxacin, survive longer than the untreated counterparts, possibly supporting the hypothesis that bacterial endotoxin is responsible for pathological changes. Furthermore the post-treatment reactive encephalopathy which follows treatment of *T. b. brucei* infected mice with trypanocidal drugs such as diminazine aceturate and which is a common feature of HAT, has been modified in mice treated with antibiotic norfloxacin (Dr. FW. Jennings, unpublished observations), suggesting that the antibiotic could be modulating a bacterial endotoxin mediated response.

The present study was designed to identify the presence in plasma of endotoxin activity during *T. b. brucei* infected mice; to establish whether it is derived from the parasite or from secondary bacterial sources; to utilise the APP for monitoring endotoxin-like activity; to demonstrate the presence of cytokines
during trypanosome infection, and to characterise the differential effects of antibiotics on CNS pathology following trypanocidal treatment.

The experiments were conducted in the chronic mouse model of trypanosomiasis as described in Chapter 1.

5.2. OBJECTIVES OF STUDY

1). To test the hypothesis that a trypanosome-derived endotoxin-like molecule(s) acting via cytokines is responsible for the pathological effects seen in the host and that secondary bacterial infection does not contribute to these effects. This was to be achieved by monitoring the acute phase proteins, SAP and Hp concentrations, packed red cell volume, endotoxin activity levels, and the presence of cytokines in plasma of trypanosome-infected mice under an antibiotic umbrella.

2). To test the hypothesis that antibiotic treatment will affect the early stage of infection by monitoring the acute phase response in frequent sampling early in infection.

3). To test the hypothesis that an antibiotic umbrella will reduce the pathology produced in T. b. brucei infected mice and, in particular, will reduce the neuropathology following diminazine aceturate induction of a post treatment reactive encephalopathy.

5.3. EXPERIMENTAL PROGRAMME

5.3.1. Study One

5.3.1.1. Part one

In study one, infected mice were treated orally with two different antibiotics. One set of mice were treated orally with polymyxin-B, an antibiotic which is not absorbed from the gastrointestinal tract, and which also binds to
lipid-A, the active moiety of LPS, thus neutralising the pathologic effects of endotoxins (Palmer and Rifkind, 1974). This treatment was designed to eliminate the GIT as the source of endotoxin in the circulation.

A second set of mice were treated with norfloxacin, a broad spectrum antibiotic, that is readily absorbed from the GIT. This acts both locally in the GIT and in the general circulation against any secondary bacterial infection which could then be eliminated as a source of endotoxin.

For each antibiotic experiment, 10 groups with 6 mice per group were used. The animals were infected with $10^4$ *T. b. brucei* parasites. The infected animals were divided randomly into two sets of 5 groups each. One set was put under oral antibiotic treatment with the drug in the drinking water from day 0 of infection. The remaining set were used as the infected, untreated control, and were given water without the antibiotic. Corresponding groups of uninfected treated and uninfected untreated groups were included as controls, and each control group was treated similarly to the infected counterparts.

In this study, 6 mice per group were sacrificed at 7 day intervals until 35 DAI, to provide plasma for the analysis of acute phase proteins (SAP and Hp), cytokines, and endotoxin-like activity, and tissues for histopathology.

5.3.1.2. Part two

Previous experiments in the infected mouse model under antibiotic cover with norfloxacin, and treated with the trypanocidal drug diminazine aceturate to induce a post treatment reactive encephalopathy, had demonstrated differences in the brain histopathology between antibiotic treated and untreated infected mice (Dr. FW. Jennings, unpublished observation).

In order to examine the effect of antibiotic treatment on the CNS pathology in infected mice treated with diminazine aceturate, a further two groups of mice in each antibiotic experiment were infected with trypanosomes.
as above. One of the groups was treated and one was not treated with antibiotic. Control groups consisting of uninfected untreated and uninfected treated mice were included. The infected antibiotic treated and the infected antibiotic untreated groups, were then given diminazine aceturate on day 31 DAI and sacrificed 38 DAI. Control groups were treated similarly. Brain tissues were taken, fixed in formalin, and processed for histopathology.

5.3.2. Study Two

Animals were treated with both antibiotics as in study one. This study involved a more frequent blood sampling from tail bleeds (2-3 day interval) to measure APP as markers of endotoxin-like action. This would highlight any differences in the APR between the 7 day sampling regime used in study one. However, the samples were of insufficient quantities for the estimation of cytokines and endotoxin-like activity.

In these experiments, for each antibiotic, twenty four mice were divided into 4 groups of 6 mice each. Two groups were infected while the other two groups acted as uninfected controls. One group from the infected and another from the uninfected group of mice were kept under the relevant antibiotic treatment from one day before infection until the termination of the experiment, 35 DAI. The remaining two groups were given water without antibiotic.

5.4. MATERIALS AND METHODS

5.4.1. Experimental Animals

Experiments were conducted on adult female inbred NIH mice weighing between 25-30 g bwt. The mice were maintained in groups of 6 per cage and fed on commercial pellets and allowed liberal access to water with or without antibiotic.
5.4.2. Trypanosomes

A strain of *T. b. brucei* GVR 35/C.1, which causes a chronic infection in mice with a survival period of about 35 days was used (Jennings and Gray, 1983; Chapter 2).

5.4.3. Drugs

The antibiotics used were polymyxin-B sulphate (Sigma chemicals company), and norfloxacin (Sigma chemicals company), a broad spectrum antibiotic. The drugs were administered in the drinking water at a dose rate of 3 mg/ml for each drug which is an equivalent dose of 0.6 mg (20 mg/kg bwt) in 0.2 ml per mouse per day, for each antibiotic.

The animals in both study one and two were kept under an alternate 4 hour light-2 hour dark regime, to facilitate maximal uptake of drug-containing water. The daily water consumption was determined each morning by measuring the amount of water in the bottles before and after topping up with fresh drug.

Diminazine aceturate was prepared in distilled water and administered ip at a dose rate of 40 mg/kg in 0.2 ml of solution.

5.4.4. Infection

Animals were infected by ip inoculation with approximately $10^4$ parasites prepared in 0.2 ml phosphate-buffered saline, pH 8.3. Control animals were injected with 0.2 ml of phosphate buffered saline.

5.4.5. Parasitaemia

Parasitaemia was monitored in the infected animals by examination of wet blood smears from tail snip at 2 day intervals in study one and at the time of blood sampling in study two.
5.4.6. Blood Sampling and Tissue Preparation

5.4.6.1. Study one

Animals were serially sacrificed at 7 day intervals starting at the seventh day after infection until 35 DAI. Five mice from each group under study, were anaesthetised and aseptically bled, intracardially. An aliquot (0.5 ml) of blood from each mouse was transferred into endotoxin free, sterile heparinised tubes containing 3.5 ml of sterile endotoxin free water, and placed in a bucket containing ice. The remaining blood from each mouse was mixed with heparin (1,000 units/ml) for the preparation of plasma. A corresponding number of uninfected controls were bled and the blood treated in a similar manner to the infected groups. The blood was then centrifuged at 2000 xg for 15 min at 4 °C to obtain plasma which was stored at -20 °C until assay for cytokines and APP. At the same time, tissue from the liver, spleen and the whole brain were taken and fixed in 10% buffered formalin for histological examination.

In the diminazine aceturate-treated animals, the brain tissues were harvested and treated in a similar manner as above at the time of sacrifice.

5.4.6.2. Study two

Each mouse was bled from the tail every 2-3 days until 35 DAI, for examination of parasitaemia, and at the same time a heparinised microcapillary tube for each mouse was filled with blood up to two third of the length. One end was sealed with plasticine and spun in a microhaematocrit centrifuge for 8 min to separate plasma. The PCV was read. The capillary tubes were then cut using a diamond marker pen, about 1 mm above the buffy coat and the plasma content expelled into an Eppendorf tube by exerting pressure on one end of the capillary tube. This plasma was stored at -20 °C until used for the estimation of SAP and Hp.
5.4.7. Biochemical Assays

5.4.7.1. Limulus amoebocyte lysate test

Endotoxin activity was measured using a quantitative chromogen LAL test (ELISA plate-end point method) (Coatest endotoxin kit; Quadratech Ltd., Epsom, Surrey UK.) as described in Chapter 2.

5.4.7.2. Acute phase protein assays

5.4.7.2.1. Serum amyloid P-component assay

Serum amyloid-P was measured using a direct ELISA utilising commercial mouse SAP as standard (Calbiochem) (Chapter 3). The primary antibody was rabbit anti-murine SAP antibody, while donkey anti-rabbit conjugated horseradish peroxidase IgG was used as the secondary antibody.

5.4.7.2.2. Haptoglobin assay

Haptoglobin was measured using the method by Suzuki and Makimura (1982) with modification by Conner et al. (1988) as described in Chapter 2.

5.4.7.3. Cytokines

Cytokine assays for IL-1, TNF-α, and IL-6 were performed at the Division of Infectious Disease Lausanne, Switzerland by Dr. D. Heumann using the bioassay methods described by Baumgartner, Heumann, Gerain, Weinbreck, Grau and Glauser (1990).

5.4.8. Histopathology

Brain, liver and spleen were taken from each mouse at the time of sacrifice, in both study one and two. These were immediately fixed in 10% buffered formalin and later processed by the routine histological procedure.
After paraffin embedding, sections of 3-4 µm thickness were cut and stained with haematoxylin and eosin (H&E) stain. The brain tissues were sectioned into three parts to reveal the fore, mid and hind brain.

5.5. STATISTICAL ANALYSIS

The data from study two and study one, were analysed using three way analysis and two way analysis of variance, respectfully. Comparisons were considered significantly different at P < 0.05.

5.6. RESULTS

5.6.1. Study One: Polymyxin-B

5.6.1.1. Parasitaemia

*T. b. brucei* parasites were demonstrated in the circulation of all infected animals by 4-5 DAI and the animals remained parasitaemic thereafter.

5.6.1.2. Endotoxin assay

The mean (±SEM) weekly plasma endotoxin levels in the trypanosome-infected and uninfected mice, with or without treatment with the antibiotic polymyxin-B are shown in figure 5.1.

In all the infected mice, with or without antibiotic treatment, plasma levels of endotoxin-like activity increased significantly (P < 0.05) by between 2-2.5 times by 7 DAI. This increased activity was demonstrated in the plasma of all infected animals at all the stages of infection. In uninfected animals, the endotoxin-like activity did not change significantly in plasma of either the antibiotic treated or untreated mice at any stage of the experiment (P > 0.05).

The mean plasma endotoxin-like activity in the infected animals ranged from 33.1 to 53.4 pg/ml compared to 10.9 to 14.8 pg/ml in the uninfected controls.
Statistical comparison of the treated and untreated animals for the effect of treatment with antibiotic polymyxin-B, indicated the antibiotic had no statistically significant effect on the levels of endotoxin activity in infected animals (P > 0.05). Similarly there was no significant difference between the endotoxin-like activity between the uninfected treated and uninfected untreated mice (P > 0.05).

5.6.1.3. Acute phase protein assay

5.6.1.3.1. Serum amyloid P-component

The mean (SEM) SAP concentrations in mice plasma of *T. b. brucei* infected, with or without polymyxin-B treatment are presented in figure 5.2. The uninfected animals showed comparable mean SAP levels and did not change significantly throughout the experimental period (P > 0.05). The mean concentrations ranged from 21.3 ± 6.7 to 33.2 ± 3.9 μg/ml and 21.1 ± 3.3 to 36.3 ± 3.0 μg/ml in the uninfected treated and uninfected untreated groups, respectively.

The infected mice showed a significant increase in the mean SAP plasma concentrations throughout the experimental period compared to the mean of the respective control samples. The mean concentrations ranged from 46.6 ± 3.5 to 122.6 ± 18.4 μg/ml and 44.2 ± 3.6 to 125.2 ± 9.7 μg/ml in the treated and untreated infected animals, respectively.

The SAP concentrations were markedly elevated on 7 and 14 DAI, but had decreased by 21 DAI and thereafter showed a gradual rise.

On statistical examination, the infected animals with or without antibiotic treatment, showed elevated mean SAP concentrations throughout the experimental period (P < 0.05). There was no significant differences due to the effect of treatment with antibiotic polymyxin-B in the infected treated when compared to the infected untreated groups (P > 0.05). Similarly in the
uninfected groups, antibiotic treatment had no significant effect on the baseline SAP concentrations (P > 0.05).

5.6.1.3.1. Haptoglobin

Haptoglobin was not detectable in the plasma of any uninfected control animals throughout the polymyxin-B experimental period. In the infected animals there was a significant increase in the concentration of haptoglobin in all samples taken throughout the experimental period. The mean concentrations are presented in figure 5.3. The mean concentrations ranged from 0.78 ± 0.07 to 1.99 ± 0.05 g/l and 0.87 ± 0.08 to 2.20 ± 0.06 g/l in the treated and untreated animals respectively (P < 0.05). The haptoglobin levels reached about 2 g/l by 7 DAI, but had decreased by about 50% by 14 DAI and remained at this level thereafter.

Comparison of the infected, treated and infected, untreated groups of animals for the effect of antibiotic treatment, indicated that Polymyxin-B had no statistically significant effect on the concentrations of haptoglobin (P > 0.05). Similarly comparison of the uninfected, treated and uninfected, untreated animals also indicated no significant differences (P > 0.05).

5.6.1.4. Cytokine assay

The cytokine levels are presented in figure 5.4. The cytokine IL-6 was detected in a few samples from the infected animals while TNF-α and IL-1 was not detectable in any of the infected animal samples. The detected IL-6 levels varied from 20-120 pg/ml, were distributed throughout the infection period and were not affected by the treatment with the antibiotic polymyxin-B. In only one sample in the uninfected, control animals was 20 pg/ml of IL-6 measured.
5.6.1.5. Histopathology

5.6.1.5.1. Liver

The livers of uninfected animals did not show any significant pathological alterations at any stage during the polymyxin-B experiment. Those from infected animals were all grossly enlarged and showed a mottled to pale appearance with occasional fatty changes. On histological examination these livers showed enlarged hepatocytes with prominent Kupffer cells. There were significant localised areas of cellular infiltration, mainly around the central vein, involving plasma cells, lymphocytes and occasionally macrophages, and in most cases this was accompanied by centrolobular necrosis (Fig. 5.5). The infiltrating cells were of the mononuclear cell type series and were more prominent at the late stages of infection. Treatment with antibiotic polymyxin-B had no significant effect on the gross pathology or histopathology of the liver tissues taken at any stage of the experiment from infected or uninfected animals.

5.6.1.5.2. Spleen

Spleens of infected mice demonstrated an increased number of active germinal centres with hyperplasia of cellular components during the early stages of infection. This was accompanied by an increase of large lymphoblasts and a predominance of plasma cells. In later stages of infection after 21 DAI, there was a reduction in the numbers of active germinal centres accompanied by a depletion of the cellular components and a relative increase in number of plasma cells and macrophages. There was no significant histological difference between spleens of polymyxin-B antibiotic treated and the untreated, infected mice.

The uninfected control mice did not show any observable histological changes irrespective of antibiotic treatment.
5.6.1.5.3. **Brain**

There were no gross or histological changes in the CNS of the uninfected mice irrespective of polymyxin-B treatment.

Brains from infected mice from 21 DAI in both the antibiotic treated and untreated mice, contained numerous trypanosomes in the choroid plexus and in a few mice this was accompanied by a slight cellular infiltrate of lymphocytes and plasma cells in the choroid plexus (Fig. 5.6). There was no significant difference between the infected animals with or without treatment with the antibiotic polymyxin-B.

In the brains of infected mice treated with diminazine aceturate 31 DAI and killed 7 DAT, there was marked cellular infiltration of the meninges and prominent perivascular cuffing (Fig. 5.7). This was mainly composed of the mononuclear cell series including lymphocytes and plasma cells. There was no significant histological differences between the brains from the antibiotic treated and untreated mice.

5.6.2. **Study One: Norfloxacin**

5.6.2.1. **Parasitaemia**

Trypanosomes were demonstrated in wet blood smears taken from the tails of infected mice by 4-5 DAI. Thereafter the parasites were detectable in the circulation of all mice unless treated with diminazine aceturate.

5.6.2.2. **Endotoxin assay**

The mean (±SEM) weekly plasma endotoxin levels in the trypanosome-infected and uninfected mice, with or without treatment with the antibiotic norfloxacin, are shown in figure 5.8.
In all the infected mice with or without antibiotic treatment, plasma levels of endotoxin-like activity increased significantly (P < 0.05) by between 2-2.5 times by 7 DAI. This increased activity was demonstrated in the plasma of infected animals throughout the experimental period. In the uninfected animals, the endotoxin-like activity did not change significantly in either uninfected treated or untreated mice at any stage of the experiment (P > 0.05).

The mean plasma endotoxin-like activity in the infected animals ranged from 32.1 to 44.4 pg/ml compared to 11.4 to 15.0 pg/ml in the uninfected controls. Statistical comparison indicated that there were no significant differences in the levels of endotoxin-like activity between the infected, treated and infected, untreated groups (P > 0.05). Similarly, there was no significant difference in the endotoxin-like levels between uninfected, treated and untreated mice (P > 0.05).

5.6.2.3. Acute phase protein assay

5.6.2.3.1. Serum amyloid P-component

The mean plasma SAP concentrations are presented in figure. 5.9. The uninfected animals showed similar mean SAP concentrations that did not change significantly throughout the experimental period (P > 0.05). The mean concentrations ranged from 21.4 ± 3.2 to 33.0 ± 3.9 μg/ml and 23.7 ± 3.1 to 30.2 ± 2.3 μg/ml in the treated and untreated groups respectively.

The infected mice showed significant increases in the mean SAP concentrations throughout the experimental period compared to their respective controls. The mean SAP concentrations were significantly elevated ranging from 50.9 ± 5.2 to 106.3 ± 14.3 μg/ml and 45.5 ± 2.9 to 121.1 ± 6.8 μg/ml in the treated and untreated groups, respectively (P < 0.05).
The higher concentrations were found in the samples taken in the early stages of infection, i.e., from 7 and 14 DAI, followed by a decrease by 21 DAI and a slight increase on 35 DAI.

Statistical analysis of the infected animals, with or without antibiotic treatment, showed significantly elevated mean SAP concentrations throughout the experimental period (P < 0.05). There were no statistically significant differences for the effect of the antibiotic norfloxacin, i.e., in the infected, treated compared to the infected, untreated group (P > 0.05). Similarly, in the uninfected groups treatment had no effect on the baseline SAP concentrations (P > 0.05).

5.6.2.3.2. Haptoglobin

The mean haptoglobin concentrations are presented in figure 5.10. Haptoglobin was not detectable in the plasma of uninfected control animals throughout the experimental period (35 DAI).

In the infected animals, haptoglobin was detectable by 7 DAI, and thereafter there were significant increases in the concentration of haptoglobin in all samples taken throughout the experimental period compared to the uninfected controls.

The mean concentrations ranged from 0.84 ± 0.14 to 1.95 ± 0.17 g/l and 1.04 ± 0.02 to 1.99 ± 0.32 g/l in the treated and untreated, infected animals, respectively. These differences were significant (P < 0.05). The haptoglobin increased to 2 g/l on 7 DAI and then fell by 50 % on 14 DAI where it remained elevated for the rest of the experimental period. From 28 DAI onwards, the infected, untreated mice plasma, had consistently higher concentrations of haptoglobin compared to the infected, treated mice.

Statistical analysis of the effect of antibiotic treatment, indicated that norfloxacin treated animals had significantly lower levels of haptoglobin
compared to the untreated groups (P < 0.05). This decrease in the levels of haptoglobin due to treatment with norfloxacin, was more pronounced during the terminal stages of infection after 21 DAI.

5.6.2.4. Cytokines assay

The cytokine levels are shown in figure 5.11. The cytokine IL-6 was only detected in some plasma samples while TNF-α and IL-1 were not detected in any samples. Infected mice IL-6 concentration varied from 20-40 pg/ml. Positive values were distributed throughout the infection period and were not affected by the treatment with the antibiotic norfloxacin. In two samples from the uninfected, control animals IL-6 was detected at the 25 pg/ml level.

5.6.2.5. Histopathology

5.6.2.5.1. Liver

The livers of uninfected animals with or without antibiotic treatment, did not show any significant pathological changes during the experiment. In contrast, those from all infected animals were grossly enlarged and showed a mottled to pale appearance with occasional fatty changes. On histological examination, these livers showed enlarged hepatocytes with prominent Kupffer cells lining the sinusoids. There were significant localised areas of cellular infiltration, mainly around the central vein involving the plasma cells, lymphocytes and occasionally macrophages, and in most cases this was accompanied by centrilobular necrosis. These changes were more pronounced during the late stages of infection.

In contrast, the livers from norfloxacin-treated mice, showed a significant reduction in the severity of cellular infiltration in the early stages of infection, before 21 DAI (Fig. 5.12), but in the later stages of infection, all mice livers were equally affected regardless of antibiotic treatment.
5.6.2.5.2. Spleen

Spleens of all infected mice with or without norfloxacin treatment demonstrated an increased number of active germinal centres with marked cellular hyperplasia, during the early stages of infection. This was accompanied by an increase in the number of large lymphoblasts and a predominance of plasma cells. In the later stages of infection, after 21 DAI, there was a reduction in the numbers of active germinal centres accompanied by a depletion of the cellular components with a relative increase in number of plasma cells and macrophages. There was no obvious histological difference due to treatment with the antibiotic norfloxacin.

The uninfected control mice did not show any observable histological changes irrespective of antibiotic treatment, but in term of gross pathology, spleens from norfloxacin-treated mice were visually relatively smaller in size compared to those from the corresponding uninfected, untreated groups.

5.6.2.5.3. Brain

There were no significant gross or histological changes in the CNS of the uninfected mice irrespective of antibiotic treatment.

The brains from infected mice sacrificed from 21 DAI, contained numerous trypanosomes in the choroid plexus especially in the lateral ventricles of the mid-brain, and in a few mice this was accompanied by a mild cellular infiltrate of lymphocytes and plasma cells. There was no significant difference in the brain histopathology between infected animals with or without antibiotic treatment.

In the brains of infected mice treated with diminazine aceturate 31 DAI and killed 7 DAT, there was marked cellular infiltration of the meninges and prominent perivascular cuffing. This was mainly composed of mononuclear
cells including lymphocytes and plasma cells. The pathology was similar in brains from antibiotic treated and untreated mice.

5.6.3. Study Two: Polymyxin-B Experiment
This study was done in order to highlight any differences that occurred, between the 7 day intervals, in the parameters monitored in study one.

5.6.3.1. Parasitaemia
Trypanosomes were demonstrated in the circulation by 4-5 DAI. All mice had high parasitaemias throughout the experimental period with fluctuating parasitaemic waves.

5.6.3.2. Packed red blood cell volume
The mean PCV values are presented in figure 5.13. The uninfected mice did not show any significant change in the mean PCV levels throughout the experimental period (P > 0.05). Treatment with the antibiotic polymyxin-B in the uninfected animals did not have any significant effect on PCV (P > 0.05).

On the other hand, the mean PCV levels in the infected animals showed a significant gradual and continuous drop from 15 DAI (P < 0.05). This reached a lowest level of 40.6 ± 1.0 and 41.1 ± 0.8% in the treated and untreated mice, respectively, 30 DAI (P < 0.001).

Comparison of the infected polymyxin-B treated and infected untreated mice showed no significant difference in the mean PCV values (P > 0.05).

5.6.3.3. Acute phase proteins assay
5.6.3.3.1. Serum amyloid P-component
The changes in the SAP concentrations are presented in figure 5.14. The plasma samples from the pre-infection and uninfected control mice had a mean
base line SAP concentrations ranging from 20.5 ± 4.8 to 37.3 ± 2.7 μg/ml throughout the experiment.

The mean plasma SAP concentrations in the infected mice started increasing shortly after infection and were significantly different from the SAP in controls by 2 DAI (P < 0.05). This rise continued, reaching peak mean concentrations of 141.4 ± 21.8 and 127.8 ± 24.5 μg/ml in the treated and untreated mice 14 and 11 DAI, respectively (P < 0.001). This was then followed by a sharp decline to levels above baseline values of between 40-50 μg/ml, at which it was maintained until termination of the experiment.

Statistical comparison of the mean SAP levels of the untreated and polymyxin-B treated infected or uninfected mice during the experiment, did not show any significant difference due to antibiotic treatment (P > 0.05).

5.6.3.3.2. **Haptoglobin**

The mean haptoglobin concentrations are presented in figure 5.15. Haptoglobin was undetectable in all samples from pre-infection and from uninfected animals. Following infection, haptoglobin was found in plasma 3 DAI. This rose to peak mean concentrations of 1.84 ± 0.25 g/l and 2.16 ± 0.14 g/l in the treated and untreated animals 6 DAI. Thereafter, the concentrations decreased to about 1 g/l where they remained for the rest of the experiment.

On statistical analysis the haptoglobin concentrations were significantly elevated throughout the experimental period in all infected mice. The antibiotic polymyxin-B had no significant effect on the mean haptoglobin concentrations in the treated groups compared to the untreated groups (P > 0.05).
5.6.4. Study Two: Norfloxacin Experiment

5.6.4.1. Parasitaemia

Parasites were demonstrated in the circulation of all infected animals by 4-5 DAI and the animals maintained a relatively high parasitaemia throughout the infection period.

5.6.4.2. Packed red blood cell volume changes

The mean PCV values during the infection are presented in figure 5.16. The mean PCV in the control mice did not show any significant changes throughout the experiment and there was no significant effect of treatment (P > 0.05). In the infected animals, there was a gradual and significant decrease in the mean PCV from 11 DAI which reached a low value of 36.2 ± 0.5 and 38.8 ± 0.9% in the treated and untreated groups, respectively, 35 DAI (P < 0.001).

There was no significant difference on the effect of treatment with norfloxacin on the mean PCV levels in the infected animals between the treated and untreated groups (P > 0.05). Similarly the antibiotic treatment had no effect on the mean PCV values of the uninfected animals.

5.6.4.3. Acute phase protein assays

5.6.4.3.1. Serum amyloid P-component

The mean SAP concentrations are presented in figure 5.17. SAP concentrations in the infected mice rose significantly by 2 DAI from baseline concentrations to reach peak mean concentrations of 122.6 ± 18.4 and 129.3 ± 13.2 μg/ml in treated and untreated mice, respectively, 14 DAI (P < 0.001). This was later followed by a decline to near pre-infection concentrations, but remaining higher than the mean of the controls, where it was maintained till termination of the experiment. Treatment of the infected mice with norfloxacin
did not have a significant effect on the SAP concentrations compared to the infected untreated groups (P > 0.05).

The uninfected animals did not show any significant change in the baseline mean concentrations of SAP with or without treatment with norfloxacin (P > 0.05).

5.6.4.3.2. Haptoglobin

The mean haptoglobin concentrations are presented in figure 5.18. The preinfection and plasma samples from the uninfected animals did not have any detectable haptoglobin. On infection, haptoglobin was detected 3 DAI and a gradual increase reached peak mean concentrations of 1.95 ± 0.17 and 2.02 ± 0.19 g/l on 10 DAI in the treated and untreated groups, respectively. This was followed by a drop to about 1 g/l 15 DAI where it remained in both infected groups of animals for the rest of the experiment. Statistical analysis on the possible effect of antibiotic treatment on the mean haptoglobin concentrations showed significant decreases in haptoglobin concentrations toward the terminal stages of infection, i.e. 28 DAI (P < 0.05), but there was no significant effect of antibiotic treatment (P > 0.05).

5.7. DISCUSSION

The results in this chapter demonstrate that infection of mice with T. b. brucei is associated with a significant increase in the levels of plasma endotoxin-like activity, anaemia, tissue pathology, and confirms the findings of Chapter 4 showing the development of an acute phase response demonstrated by increases in the plasma concentrations of the acute phase proteins, SAP and haptoglobin. The endotoxin-like activity increased significantly (200%) by 7 DAI in all infected mice in study one, and remained elevated throughout the experiment. The endotoxin-like activity was not affected by treatment with the
antibiotics polymyxin-B and norfloxacin. This suggests that the endotoxin-like activity is of parasite origin and not from bacteria or absorption from the GIT.

The APP in plasma of infected mice in studies one and two, increased to peak levels by 10-15 and 7-10 DAI, for SAP and Hp, respectively. Thereafter in both studies, SAP decreased to just above baseline control levels and remained low for the remaining part of the experiment. Haptoglobin, on the other hand, decreased to around 50% of the peak concentration, and remained elevated for the rest of the experimental period.

The plasma concentration of the APP in the mice treated with polymyxin-B in studies one and two were not affected by the treatment with the antibiotic. Polymyxin-B which not only kills gastrointestinal tract bacteria but also binds to bacterial LPS preventing absorption, virtually eliminating the gastrointestinal tract as a source of endotoxin, had no significant effect on the development of the APP. Treatment with norfloxacin in both studies, did not affect the concentrations of SAP in plasma of infected mice, but plasma from infected treated mice had slightly lower Hp concentrations compared to the infected untreated groups in study one, an effect that was more pronounced during the later stage of infection, i.e., 28 DAI. This finding was not repeated in study two where there was no significant differences in haptoglobin concentrations between infected, untreated and infected norfloxacin treated mice. It is probable that the slight fall in study one was artificial, although a contribution from secondary bacterial infection can not be totally dismissed.

Indeed serial measurements of CRP in leukaemia (Mackie et al, 1979; Schofield et al, 1982; Walker et al, 1984) and systemic lupus erythematosus (Becker et al, 1980) were found to be higher in cases with secondary bacterial infections compared to the uncomplicated cases. Also the CRP concentrations in these cases of intercurrent bacterial infections were found to decrease
following effective antibacterial treatment but remained elevated in the unresponsive cases (Philip, 1982; Schofield et al, 1982).

The only effect of antibiotic treatment shown by histological examination of the tissues, was in the norfloxacin treated mice where a decrease in the cellular infiltration of the liver was observed during the early stages of infection, before 21 DAI. However after this all tissues were equally affected with or without antibiotic treatment, further evidence that bacterial products are unlikely to be responsible for the trypanosome pathology.

The presence of a toxin-like molecule in trypanosomes has been postulated for a long time (Tizard et al, 1978) and may be responsible for initiating the pathogenesis of African trypanosomiasis (Pentreath, 1994). Indeed, endotoxin-like activity has been demonstrated by the LAL method in trypanosome lysate and elevated levels have been reported in the serum of mice infected with trypanosomes (Alafiatayo et al, 1993).

In previous experiments, the origin of this toxin in different parasitic infections including trypanosomiasis, and its pathological significance has not been elucidated. Notably, the occurrence of secondary bacterial infection and the absorption of endotoxin from the gastrointestinal tract of infected host following damage to the intestinal wall during infection, an observation documented in T. cruzi infection (Bambirra et al, 1984) have been possible sources of the activity demonstrated in trypanosomiasis (Alafiatayo et al, 1993).

In T. cruzi, a close relative of the parasite causing African trypanosomiasis, an endotoxin-like lipopolysaccharide molecule has been demonstrated (Goldberg et al, 1983). Although endotoxins have been extensively studied in bacteria, molecules with endotoxin-like activity have also been demonstrated in other micro-organisms including Plasmodium falciparum (Jakobsen et al, 1988), Sarcocystis cruzi (Fayer, 1988), and Chlamydia (Lewis
et al, 1979). Presumably, all these endotoxin-like activities operate in vivo by activation of the cytokine network which is discussed below.

These results suggest that the endotoxin-like activity measured in plasma of infected mice is of trypanosome origin. This is based on the observation that when animals were treated with antibiotic throughout the infection, the endotoxin-like activity in plasma was not diminished in comparison to infected untreated animals. Although tissue damage is known to be an inducer of cytokines and thus the APR (Whicher and Dieppe, 1985; Mozes et al, 1989), the possibility that the observed APR was due to the tissue damage resulting from the infection and the invasiveness of T. b. brucei is unlikely. This is not only from the result of Chapter 4 where a tissue invasive and non-invasive trypanosome species caused an APR, but also because the protein concentrations of Hp and SAP, started rising before parasites were demonstrable in circulation and most significantly when little or no tissue pathology was observed. The highest response of both proteins in the infected animals was observed during early infection, i.e., 7-10 DAI, whereas, during late infection when maximum tissue pathology was observed, the levels of the acute phase proteins had decreased by 50% for Hp and to near baseline levels for SAP. It is of interest that macrophages, which are known to be a main source of cytokines, from trypanosome-infected animals during early infection, show increased cytokine production (Sileghem, Flynn, Saya and Williams, 1993), while in late infections they show a decreased ability to produce cytokines (Mitchel, Pearson and Gouldie, 1986) and prostaglandins (Fierer et al, 1984), although species differences have been reported (Sileghem et al, 1989).

In addition, non-invasive trypanosomes such as T. congolense that produce minimal tissue damage cause the production of pro-inflammatory molecules including cytokines (Mitchel et al, 1986; Sileghem et al, 1993),
prostaglandins (Mutayoba, Meyer, Osaso, and Gombe, 1989), and also APP (Thomson et al, 1973). *Trypanosoma congoense* infection has also been shown to induce an increase in TNF-α receptor expression on peripheral blood leukocytes in sheep (Winstanley, Holmes, Katunguka-Rwakishaya, Perkins, Fishwick and Murray, 1993).

Furthermore whole viable trypanosome and non viable trypanosome lysates have been documented to be able to stimulate the production of cytokines *in vitro* (Hotez et al, 1984; Gichuki, 1994; Pentreath, 1994) and *in vivo* (Oka et al, 1989). This suggests that cytokine production is not dependent on an active infection but is possibly due to a host response to parasite derived product(s) such as the endotoxin-like activity identified here.

The decrease in the concentrations of the APP, in spite of the elevated endotoxin-like activity which is maintained from 7 to 35 DAI, might be explained by reduction in endogenous production of the pro-inflammatory mediators during trypanosomiasis (Fierer et al, 1984; Mitchel et al, 1986), an observation also noted during continuous exposure to sub-lethal doses of gram negative bacterial endotoxin (Flohe, Heinrich, Schneider, Wendel and Flohe, 1991; Roth et al, 1994). Indeed, mice made tolerant to lethal doses of endotoxin by continuous injection with sublethal doses of *Escherichia coli* LPS before infection, showed increased survival time and were able to withstand high parasitaemia, following infection with *T. b. brucei*, *T. congoense*, *T. duttoni* and *T. rhodesiense* (Singer et al, 1964) suggesting a possible similarity in the mechanism of action between trypanosomes and LPS.

The fact that only minor changes in APP and liver pathology were observed in norfloxacin-treated mice, suggests that secondary bacterial infection, is not a major factor in the pathogenesis of trypanosomiasis. However, the pathological changes during the later stages of trypanosome infection such as meningoencephalitis, could be exacerbated by secondary
bacterial infection (Mott, 1907). Secondary bacterial infection is a common cause of death in chronic infections (Goodwin, 1970). The results also suggest that endotoxin from the gastrointestinal tract, is not important to the endotoxin-like activity during trypanosomiasis as treatment with polymyxin-B, an antibiotic known to have antibacterial effect and the ability to bind to and inactivate endotoxin (Palmer and Rifkind, 1974; Corrigan and Bell, 1971), did not have any effect on the levels of APP, endotoxin-like activity, or the tissue pathology.

The nature of the molecule(s) responsible for the increased endotoxin-like activity is not clear. Although the LAL assay for endotoxin is assumed to be lipid-A specific, it has also been shown to react with other non lipopolysaccharide molecules, including, polynucleotides and proteins (Elin and Wolff, 1973), Mycoplasma lipoglycans (Weinberg, Smith and Kahane, 1980) and antigens from gram positive bacteria (Brunson and Watson, 1976; Baek, Hoiby, Hertz and Espersen, 1985). The chemical nature of the trypanosome molecule(s) responsible for the LAL reaction and/or induction of the APR needs to be determined in order to understand the mechanism behind the production of the pathological changes and the APR during trypanosome infection.

Only low amounts of IL-6 were detected in occasional samples in study one. This is probably due to the length of time between sampling which was 7 days and is also likely to have resulted in missing raised levels of IL-1 and TNF-α as cytokines have short plasma half-lives resulting from enzymatic degradation, tissue binding (Beutler, Milsark and Cerami, 1985), or as a result of local compartmentalisation (Nelson, Bagby, Bainton, Wilson, Thomson and Summer, 1989; Waage et al, 1989). The absence of cytokines in the circulation, though the biological activity can be demonstrated, has been documented in mice (Hinshaw, Tekamp-Olson, Chang, Lee, Taylor, Murray, Peer, Emerson,
Passey and Kuo, 1990). The investigation of circulating cytokines in *T. b. brucei* was limited here to the assay of the weekly samples taken in study one. To provide more conclusive results, samples should be taken more frequently. A problem might arise because of the small volume of plasma which could be taken. Alternatively a large number of animals could be sacrificed. Such investigations might provide useful information.

APP can act as markers of combined cytokine activity. They remain in the circulation for at least 24 hours and also represent the systemic outcome of the complex interaction of the cytokine network within the host. IL-6, a multifunctional cytokine known to activate T and B-cells (Kishimoto and Hirano, 1988), is the major regulating cytokine for the hepatic APP synthesis (Heinrich et al, 1990). It is likely that IL-6 was responsible for the stimulation of the observed APR but its production has been shown to be initiated by other cytokines including TNF-α and IL-1 (Heinrich et al, 1990) but these were at an undetectable level. Similarly in horses treated with endotoxin, TNF-α elevation has been shown to precede that of IL-6 (Mackey and Lister, 1992) and in rats, repeated doses of endotoxin resulted in repeated IL-6 formation but reduced TNF-α formation (Flohe et al, 1991).

In the infected animals treated with diminazine aceturate, severe pathological changes were observed which were confined to the CNS. However the severity of the lesions was not affected by the antibiotic treatments.

This is the first study to link trypanosome endotoxin-like activity, cytokine IL-6, the APP response, and the pathological changes observed during African trypanosomiasis. It suggests that trypanosomes may cause pathological alterations in infected hosts by releasing active molecule(s) with endotoxin-like activity, which lead to the production of cytokines and the development of the APR observed during trypanosomiasis.
5.8. CONCLUSION

These results demonstrate that infection of mice with trypanosomes causes a significant increase in the plasma levels of endotoxin-like activity as well as the acute phase proteins, SAP and haptoglobin. This is also accompanied by increased levels of cytokine IL-6. Treatment of infected mice with the antibiotics norfloxacin or polymyxin-B, to eliminate any other secondary source of endotoxin including secondary bacterial infection and absorption from the gastrointestinal tract, did not have any significant effect on these parameters except a slight modification of liver pathology and minor reduction in haptoglobin concentrations in the terminal stage of infection. This suggests that the trypanosomes and not bacteria were responsible for the pathological changes, possibly by producing during the infection, a molecule(s) with endotoxin-like activity.
Fig. 5.1. Weekly mean (± SEM) changes in the plasma endotoxin levels in *Trypanosoma brucei brucei*-infected and control mice with or without polymyxin-B antibiotic treatment.

Fig. 5.2. Weekly mean (± SEM) changes in the plasma concentrations of serum amyloid P-component in *Trypanosoma brucei brucei*-infected and control mice with or without polymyxin-B antibiotic treatment.

Fig. 5.3. Weekly mean (± SEM) changes in the plasma concentrations of haptoglobin in *Trypanosoma brucei brucei*-infected and control mice with or without polymyxin-B antibiotic treatment.
Fig. 5.4. Weekly levels of cytokine IL-6 in *Trypanosoma brucei brucei*-infected mice with (A) or without (B) polymyxin-B treatment. Six samples were analysed at each time point for IL-6 concentration, only samples containing a detectable concentration of IL-6 are shown on the figure.
**Fig. 5.5 a and b.** Liver of *Trypanosoma brucei brucei*-infected mouse killed on day 35 DAI showing severe cellular infiltration in the portal area (C) and the parenchyma (D) with hepatocyte necrosis (N). Haematoxylin and eosin. x100 and x80.
**Fig. 5.6.** Brain of *Trypanosoma brucei brucei*-infected mouse killed on day 35 DAI. Note the large number of trypanosomes in the choroid plexus (T). Haematoxylin and eosin. x100.

**Fig. 5.7.** Cerebrum of *Trypanosoma brucei brucei*-infected mouse treated with diminazine aceturate 31 DAI and killed 7 DAT. Note the severe meningitis (M) and perivascular cuffing (C). Haematoxylin and eosin. x80.
**Fig. 5.8.** Weekly mean (± SEM) changes in the plasma endotoxin levels in *Trypanosoma brucei brucei*-infected and control mice with or without norfloxacin antibiotic treatment.

**Fig. 5.9.** Weekly mean (± SEM) changes in the plasma concentrations of serum amyloid P-component in *Trypanosoma brucei brucei*-infected and control mice with or without norfloxacin antibiotic treatment.

**Fig. 5.10.** Weekly mean (± SEM) changes in the plasma concentrations of haptoglobin in *Trypanosoma brucei brucei*-infected and control mice with or without norfloxacin antibiotic treatment.
Fig. 5.11. Weekly levels of cytokine IL-6 in *Trypanosoma brucei brucei*-infected mice with (A) or without (B) norfloxacin treatment. Six samples were analysed at each time point for IL-6 concentration, only samples containing a detectable concentration of IL-6 are shown on the figure.
Fig. 5.12. Liver sections of *Trypanosoma brucei brucei*-infected mouse killed on day 21 post infection. Infected norfloxacin treated (Fig. 5.12a) and infected untreated (Fig. 5.12b). Note the reduced centrilobular cellular infiltration in the antibiotic-treated and compared to the untreated mice. Haematoxylin and eosin. x50.
Fig. 5.13. Mean (± SEM) changes in the packed red cell volume (PCV) in *Trypanosoma brucei brucei*-infected and control mice with or without polymyxin-B antibiotic treatment.

Fig. 5.14. Mean (± SEM) changes in the plasma concentrations of serum amyloid P-component in *Trypanosoma brucei brucei*-infected and control mice with or without polymyxin-B antibiotic treatment.

Fig. 5.15. Mean (± SEM) changes in the plasma concentrations of haptoglobin in *Trypanosoma brucei brucei*-infected and control mice with or without polymyxin-B antibiotic treatment.
Fig. 5.16. Mean (± SEM) changes in the packed red cell volume (PCV) in *Trypanosoma brucei brucei*-infected and control mice with or without norfloxacin antibiotic treatment.

Fig. 5.17. Mean (± SEM) changes in the plasma concentrations of serum amyloid P-component in *Trypanosoma brucei brucei*-infected and control mice with or without norfloxacin antibiotic treatment.

Fig. 5.18. Mean (± SEM) changes in the plasma concentrations of haptoglobin in *Trypanosoma brucei brucei*-infected and control mice with or without norfloxacin antibiotic treatment.
CHAPTER 6
LIPOPOLYSACCHARIDE AND LIPID-A IN TRYPANOSOME LYSATES AND MEMBRANE-ENRICHED TRYPANOSOME FRACTIONS
6.1. INTRODUCTION

In African trypanosomiasis attempts to develop a vaccine as a means of controlling the disease have been hampered by the ability of the parasite to evade the immune system of the host through antigenic variation of the surface coat (Vickerman, 1978; Nantulya, 1986).

Once established in the host, the parasite causes a variety of immunological and non-immunological changes eventually leading to death (Murray et al, 1982; Molyneux et al, 1984). Although the pathological changes following trypanosome infection have been well described in infected hosts, the underlying pathogenic mechanisms are not clearly understood (Pentreath, 1991). This has led to several hypotheses being put forward to explain the observed pathological alterations, which include the immune-mediated responses (Mansfield, 1990) and trypanosome-derived toxins (Tizard et al, 1978). Observations described in the previous chapters of this thesis support the hypothesis that trypanosomes can produce an endotoxin-like molecule(s) capable of stimulating the host defence system.

The pro-inflammatory molecules such as cytokines and interferons have been shown to produce, at least in part, the pathological changes observed during trypanosomiasis and other parasitic diseases (Titus et al, 1991; Jakobsen, Bate, Tavern and Playfair, 1995). During trypanosomiasis increased production and release of cytokines have been documented (Hunter et al, 1992a; Reincke et al, 1993) and trypanosomes or their products have been shown to have the ability to induce the production of cytokines from different cell types in vitro (Hotez et al, 1984; Olsson et al, 1991; Gichuki, 1994; Alafiatayo et al, 1994).

In trypanosomiasis, these pro-inflammatory molecules are thought to be responsible for initiating the inflammatory and non-inflammatory changes in the infected hosts (Rouzer and Cerami, 1980; Bancroft et al, 1983; Hunter et al, 1991; Hunter et al 1992a), but the precise mechanisms or trypanosome
molecule(s) involved in their production are not well understood (Pentreath, 1991). Trypanosomes are known to produce factor(s) that cause peritoneal cells to produce mediators with lipoprotein lipase enzyme suppressive activity (Hotez et al, 1984), an effect known to be mediated by TNF-α. Trypanosomes are also documented to release molecule(s) that inhibit human promyelocytic HL-60 cell growth (Keku, Seed, Sechelski and Balber, 1994), and prevent the progression of activated human T-lymphocytes through the cell cycle (Sztein and Kierszenbaum, 1991). A trypanosome product, trypanosome lymphocyte triggering factor (TLTF) has been purified from T. b. brucei and has been demonstrated to activate CD8+ T cells to release interferon-γ (Olsson et al, 1991). The chemical nature of this molecule(s) is not known although the possible presence of a trypanosome toxin has been speculated (Tizard et al, 1978). Membrane fractions of trypanosomes injected into mice have been shown to mimic the immunosuppressive and mitogenic effects of the living parasite (Clayton et al, 1979). Indeed the possible production of a trypanosome mitogen and its involvement in immunosuppression through non-specific stimulation and multiplication of B-lymphocytes, preventing their participation in immune responses has been suggested (Urquhart et al, 1973; Murray et al, 1974b). This mitogen is also postulated to be involved in the production of the marked increase in serum immunoglobulins, particularly IgM, and the production of heterophile antibodies and auto-antibodies characteristically found in trypanosomiasis (Greenwood, 1974). Recent work by several workers (Alafiatayo et al, 1993) and the observations in this thesis, have demonstrated increased endotoxin-like activity in plasma from trypanosome-infected mice. In addition, trypanosome lysates showed high activity in the LAL assay for endotoxin activity, although the molecule(s) responsible for this activity has not yet been characterised (Alafiatayo et al, 1993).
As discussed earlier, the involvement of toxin-like molecules in parasitic infections has been suggested in the pathogenesis of other parasitic diseases including malaria (Jakobsen et al, 1988), *Chlamydia* (Lewis et al, 1979), *Sarcocytis* (Fayer, 1988) and Chagas' disease (Goldberg et al, 1983). In *Trypanosoma cruzi* (Chagas' disease) infections, the toxin has been characterised and shown to be a lipopolysaccharide molecule (Goldberg et al, 1983; de Simone, Pinho, Vanni and de Carvalho, 1991).

The most characterised toxin with similar activity is the LPS molecule of gram negative bacteria. This is known to consist of two distinct parts, the outer core containing polysaccharide and an inner core containing the lipid-A moiety (Luderitz, Freudenburg, Galanos, Lehmann, Rietschel and Shaw, 1982). The lipid-A moiety is a highly conserved part of the LPS molecule in the different species of the genera (Mutharia, Crockford, Bogard and Hancock, 1984; Bogard, Dunn, Abernethy, Kilgarriff and Kung, 1987). Most of the immunogenic and pathogenic activity of such bacterial LPS is known to be the property of this lipid-A moiety (Rietschel, Brade, Brandenburg, Flad, de Jong-Leuveninck, Kawahara, Lindner, Loppnow, Luderitz, Schade, Seydel, Sidorczyk, Tacken, Zahringer and Brade, 1987; Miller and Hjelle, 1990). Gram negative bacteria LPS is one of the most potent inducers of cytokines and other pro-inflammatory molecules. Indeed during bacterial infections, the release of cytokines has been associated with the harmful effects of endotoxin (Saukkonen et al, 1990; Sharief et al, 1992). The use of LPS inhibitors such as antibodies and chemicals that bind LPS has been documented to counteract the pathological effects of endotoxin and reduce cytokine release (Baldwin et al, 1991).

The study described in the previous chapter demonstrated in plasma of *T. b. brucei*-infected mice the presence of endotoxin-like activity which was not
eliminated by antibiotic treatment and was therefore likely to be derived from the parasite.

In order to further our understanding of the pathogenesis of trypanosomiasis it is necessary to characterise the molecule(s) producing the endotoxin-like activity. Greater understanding of the nature of molecule(s) in trypanosomes that are directly involved in the production of the pathological effect of the parasite would be of value in development of therapy and could be useful candidate antigens for vaccine production.

6.2. OBJECTIVES OF THE STUDY

1). To identify and quantify endotoxin-like activity in trypanosome lysates and protein rich trypanosome membranes using the LAL assay.

2). To establish whether the endotoxin-like activity of trypanosomes is derived from compounds similar to those in the lipopolysaccharide of gram negative bacteria. This was attempted with a highly sensitive silver stain for LPS and by Western blotting with a monoclonal antibody to Lipid-A.

6.3. EXPERIMENTAL PROGRAMME

This study involved the measurement of endotoxin levels and electrophoretic characterisation of trypanosome lysates and protein rich membranes for the presence of LPS and/or a lipid-A moiety. The sodium dodecyl sulphate polyacrylamide gel electrophoresis method (SDS PAGE) used to study different proteins in complex mixtures, has been adapted for the study of bacterial LPS (Davies, Ali, Parton, Coote, Gibbs and Freer, 1991; Davies, Parton, Coote, Gibbs and Freer, 1994). The method separates different molecules according to their molecular radius. The molecules can be visualised by appropriate staining techniques or can be transferred on to nitrocellulose membranes for Western blotting and identified with appropriate antibodies.
(Towbin et al, 1979; Mutharia et al, 1984). For the study of LPS, a very sensitive silver stain method has been widely used to demonstrate low levels of LPS (Tsai and Frasch, 1982). Modifications of the conditions such as temperature and concentrations of reagents, ensures that LPS is stained and not proteins (Hitchcock and Brown, 1983). This can further be improved by the use of proteinase K to digest proteins in LPS samples (Davies et al, 1991).

In this study endotoxin activity was assessed by use of the LAL assay which is believed to react with lipid-A of LPS while Western blotting was carried out using an anti lipid-A monoclonal antibody (Mutharia et al, 1984; Dunn, Warren, Bogard and Cerra, 1985). The SDS-PAGE gels were stained by the silver stain method as described by Tsai and Frasch, (1982).

6.4. MATERIAL AND METHODS

6.4.1. Animals

6.4.1.1. Rats

The rats used to expand trypanosome populations were Sprague Dawley white males (500-600 g). These yield 10-15 ml of blood by intracardial bleeding.

6.4.2. Trypanosome

*Trypanosoma brucei brucei* stabilate TREU-226 raised in rats was used as the source of trypanosome products. This causes an acute infection in rats leading to death 5-7 days after infection.
6.4.3. Endotoxin Controls

6.4.3.1. Lipopolysaccharide

LPS from *Escherichia coli* 0111:B4 prepared by water phenol extraction procedure was obtained from Sigma Chemicals, Dorset UK. and was used as the LPS positive control.

6.4.3.2. Lipid-A

Lipid-A was purchased from Calbiochem-Behring Corporation La Jolla, California. This lipid-A was derived from *Salmonella minnesota* bacteria by extraction using the water phenol procedure and was used as the lipid-A positive control. Endotoxin-free distilled water was used as the negative control.

6.4.4. Proteinase K

Proteinase K was purchased from Sigma Chemical Company St. Louis USA. and was from *Tritirachium album* (EC 3.4.21.14), containing 18 mg/ml protein, 330 units/ml in 40 % (v/v) glyceral, 10 mM Tris-HCl, pH 7.5, and 1 mM calcium acetate.

6.4.5. Monoclonal Antibody

Monoclonal antibody 8A1, was a generous gift from Centorcor (Malvern, Pennsylvania. USA.) and was raised from mice spleen cells harvested from animals immunised intraperitoneally with heat-killed *E. coli* J5 LPS and then boosted intravenously with a mixture of *E. coli* J5 LPS and *S. minnesota* Re LPS. Three days after the immunisation spleen cells were harvested and fused with the NS-1 murine myeloma cell line (Bogard *et al*, 1987). The 8A1 monoclonal antibody has been demonstrated to have a wide range of cross-
reactivity with many species of gram negative bacteria and shows strong and specific binding to lipid-A (Mutharia et al, 1984; Dunn et al, 1985).

6.5. PARASITOLOGICAL TECHNIQUES

6.5.1. Infection

Rats were infected by intraperitoneal inoculation with approximately $10^6$ trypanosomes in 0.2 ml of phosphate saline buffer containing 15% glucose (PBSG), pH 8.0.

6.5.2. Monitoring Parasitaemia

The parasitaemia was monitored by tail bleeds and examined under the microscope. The blood was collected from the rats at peak parasitaemia.

6.6. BLOOD SAMPLING AND TRYpanosome Separation

6.6.1. Blood Sampling

Heparinised blood was collected aseptically by intracardiac puncture from infected rats at the peak parasitaemia. The rats were terminally anaesthetised using carbon dioxide and bled by intracardiac puncture using a 27 gauge needle and 10 ml syringe.

6.6.2. Trypanosome Separation

All buffers, other fluids and apparatus used were either endotoxin free or were rendered endotoxin free by autoclaving for 4 hr at 180° C and for the apparatus this was followed by 2 hr in dry heat at 100° C.

Trypanosomes were separated from blood with diethylaminoethyl (DEAE-52) cellulose anion exchange columns (Whatman Paper Ltd, Spring Mill, Kent, England) by the method of Lanham (Lanham, 1968; Lanham, Williams and Godfrey, 1972). The separating column consisted of a slit-sieve
funnel, rubber tubing, retort stand and clamp, tubing tip and pasteur pipette. The apparatus was assembled by clamping the funnel on the stand, and at the end of the funnel, rubber tubing with an attached pasteur pipette was connected.

The separating buffer (PBS) consisted of di-sodium hydrogen phosphate (Na$_2$HPO$_4$-13.48 gm), sodium di-hydrogen phosphate (NaH$_2$PO$_4$. 2H$_2$O-0.78 gm), and sodium chloride (NaCl-4.25 gm), made up to 1 litre with distilled water (H$_2$O), and the pH adjusted to pH 8.0. The PBS was mixed with distilled water at a ratio of 6:4, and into each litre of buffer water mixture, was added 15 gm of glucose just prior to use (PBSG).

The DEAE-52 cellulose was prepared by suspending the pre-swollen DEAE-52 in PBS while gently stirring to avoid the formation of fine particles. The pH was adjusted to pH 8.0 using dilute orthophosphoric acid. The cellulose was then allowed to settle, after which the supernatant was poured off and more buffer added. This washing procedure was repeated at least twice before use, to remove fine particles.

The separating buffer was poured into the assembled funnel and air bubbles expelled by gently tapping the side of the funnel. A filter paper (Whatman No. 1) was placed on the surface of the buffer and by slowly allowing the buffer to run through the funnel, the filter paper tightly fitted onto the grid by suction pressure. Into this, the prepared separating cellulose was poured gently and packed to the required depth by adding buffer and allowing it to run through several times.

Heparinised blood from infected animals was carefully and evenly spread on the surface of the packed column. The buffer was then allowed to run through the column and collected into a beaker with continuous monitoring by light microscopy for the appearance of trypanosomes. The trypanosomes and buffer were then collected into centrifugation tubes, placed on ice, and centrifuged at 3,500 xg at 4° C for 20 min. The supernatant was carefully
discarded and fresh buffer was added to the pellet and the centrifugation procedure repeated again. This washing process was repeated at least three times before the trypanosomes were used. The trypanosomes collected at the bottom of the tubes as a pellet, were stored at -20°C.

6.7. PREPARATION OF TRYPANOSOME PRODUCTS

6.7.1. Whole Trypanosome Lysate

For the preparation of trypanosome lysate, whole trypanosomes were disrupted by repeated freeze-thawing. The trypanosomes in tubes were placed in liquid nitrogen for 10 min and then allowed to thaw at room temperature followed by vortexing and repeatedly passing through a fine 26 gauge needle. This procedure was repeated until no entire trypanosomes could be observed under the microscope.

6.7.2. Membrane-Enriched Trypanosome Fraction

The preparation of membrane enriched fraction from the trypanosome, was performed by the method of Rovis and Baekkeskov (1980). All the steps were performed at 0-4°C.

Two ml of packed volume of the separated trypanosomes was re-suspended in 10 ml of PBSG in a 30 ml centrifuge tube into which was added 2 ml volume equivalent of solid glass beads that had been pre-rinsed with PBSG. The mixture was homogenised in a motor for at least 2 min, maintaining the tube on ice. Into this homogenate was added 10 ml of HEPES sucrose mixture (10 mM 4-(hydroxyethyl)-1-piperaziny1-ethane-2-sulphonic acid, pH 7.4, 0.25 M sucrose, 1 mM ethylenediamine tetra acetic acid (EDTA), 1 mM benzamidine, 1 mM aprotinin).

A sample was then taken and examined under the microscope for the presence of entire trypanosomes. If any entire trypanosomes were observed, the
The homogenisation procedure was repeated. The homogenate was then spun for a few seconds at 4°C to pellet the beads and the supernatant collected and kept on ice. Into the beads was added 5 ml of HEPES/sucrose and spun again for a few seconds and the supernatant collected. This extraction of the beads was repeated two more times.

The different supernatants were pooled and centrifuged at 5,000 xg for 20 min at 4°C, and the pellet containing mainly the nuclei and unbroken cells discarded.

The supernatant was then centrifuged at 50,000 xg at 4°C, for 50 min and the supernatant was discarded. The pellet obtained was re-suspended in 25 ml of HEPES/sucrose and the centrifugation procedure repeated as above.

The pellet (membrane-enriched fraction) was re-suspended in 2.5 ml HEPES/magnesium sulphate buffer, pH 8.6 (1 mM HEPES, 1 mM magnesium sulphate, 1 mM benzamidine and aprotinine), per ml of starting packed cell volume and transferred to a dialysis bag and dialysed for 20 min against 1 litre of cold HEPES/magnesium sulphate containing benzamidine and aprotinine.

A 26% (w/v) dextran solution (Sigma Chemical Company) in HEPES/magnesium sulphate buffer was prepared, and 11 ml of this was placed into a swing-out tube and overlaid with 2.5 ml of the dialysed membrane-enriched fractions. This was centrifuged at 35,000 xg in a swing-out rotor for 1.3 hr. This separates the plasma membranes from the golgi, endoplasmic reticulum and lysosomes which are retained by the dextran. The top layer was carefully removed, made up to 30 ml with HEPES/sucrose buffer and spun at 48,000 xg for 1 hr. The pellet was re-suspended in 0.5-2 ml in HEPES/sucrose containing protease inhibitors. The concentration of protein was then measured by the biuret method as described in Chapter 2, and the samples stored at -70°C.
6.8. BIOCHEMICAL ASSAYS

6.8.1. Endotoxin Assay

Endotoxin activity was measured using the quantitative chromogenic Limulus amoebocyte lysate test (LAL test) (Coatest Endotoxin Kit; Quadratech Ltd) as described in Chapter 2.

The standards were prepared to cover the range 0.15-1.2 EU/ml and the trypanosome lysate and trypanosome protein rich membrane were used as the starting materials.

6.8.2. Protein Assay

The protein content of the trypanosome products was estimated by the biuret method utilising a automated analyser (Cobas Mira).

6.9. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

Samples were solubilized in an equal volume of lysis buffer containing 4% dodecyl sulphate lauryl sulphate (SDS), 10% DL-dithiothreitol (DDT), 20% glycerol, 1 M Tris-Cl (pH 6.8), 40 mM EDTA, and 0.01% bromophenol blue. The lysates were then heated at 100° C for 10 min.

For protein digestion, 25 µg of proteinase-K (PK) (Sigma Chemical Company) solubilized in 10 µl of lysis buffer was added to each boiled lysate and incubated at 60° C for a further 60 min.

Where whole trypanosome or membrane pellets were used, the pellet was either solubilized with an equal volume of lysis buffer and thereafter treated as the lysates above or was mixed with lysis buffer containing the PK and incubated at 60° C for 60 min.

As well as trypanosome products, control material consisting of bacterial LPS (48 EU/ml) and lipid-A (5 ng) preparations was subjected to SDS-PAGE
electrophoresis with and without proteinase K digestion, using the discontinuous SDS-PAGE system as described by Laemmli (1970). Electrophoresis was performed in a vertical slab gel apparatus (SE-250) (Hoeffer Scientific Ltd., San Francisco, USA), in buffer comprising 25 mM Tris-HCl and 192 mM glycine (pH 8.3). The slab gel (140 x 100 x 0.75 mm) incorporated a 4% acrylamide stacking gel and 15% acrylamide separating gel.

Samples were subjected to electrophoresis at 20 miliamperes (mA) per gel until the bromophenol blue migrated to the edge of the tank buffer. The gels were prepared in duplicate and one was stained by the silver stain method and the other used for immunoblot transfer.

Effectiveness of protein transfer in the immunoblot was demonstrated by staining the gels from which protein had been transferred by the Coomassie blue stain method.

6.10. STAINING TECHNIQUES

6.10.1. Silver Stain

The gels were stained using the LPS sensitive silver stain method of Tsai and Frasch (1982). This staining method preferentially stains for LPS and not proteins by including 29.4% ammonium hydroxide in the staining reagent and keeping the staining solution at an optimal temperature of 25 °C (Hitchcock and Brown, 1983).

After electrophoresis, the LPS on the gel was fixed by placing the polyacrylamide gels in a clean glass dish containing 200 ml of a 40% ethanol-5% acetic acid solution and incubating overnight. The solution was then replaced with 0.7% periodic acid in 40% ethanol-5% acetic acid solution to oxidise the LPS for 5 min. This was followed by three, 15 min washes in a second container with 500-1000 ml distilled water per wash. The water was drained off and 150 ml of freshly prepared staining reagent (2 ml concentrated
ammonium hydroxide (29.4%), 28 ml of 0.1 M sodium hydroxide, 5 ml of 20% silver nitrate (w/v), and 115 ml distilled water) was added and kept under continuous vigorous agitation for 10 min. At the end of the above procedure, the staining solution was decanted and the gels washed three times in distilled water for 10 min per wash as above. The gels were then developed by replacing the water with 200 ml formaldehyde developer solution containing 50 mg citric acid and 0.5 ml formaldehyde per litre. Development was stopped when the stain reached the desired intensity or the clear gel showed the first sign of discoloration. The gels were then washed and stored in distilled water or dried using a vacuum pump dryer as described in section 6.10.3.

6.10.2. Coomassie Stain

The gels were stained in Coomassie blue stain (0.125% Coomassie blue in 50% methanol, 10% acetic acid) for 30 min after which they were washed with destaining solution 1 (50% methanol, 10% acetic acid) for 5 min and later this was decanted and replaced with destaining solution 2 (5% methanol and 7% acetic acid). The gels were allowed to destain to give minimal background colour of the gels while retaining an intense protein stain. The gels were then dried as described in section 6.10.3.

6.10.3. Gel Drying

The gels were dried under vacuum at a temperature of 80° C for 1 hr using a vacuum pump (Biorad). Prior to drying, the gels were soaked in 500 ml of a 3% glycerol (w/v), 40% methanol, 10% ethanol solution for about 3 hr with constant gentle agitation on a rotary table to ensure crack-free drying. A final 10 min soak in a 3% glycerol (w/v) solution was performed to rehydrate them back to their original size before drying.
A fresh dry sheet of 3 MM filter paper (Whatman) was cut to the appropriate size and placed on the drying apparatus. The gels were placed on the filter paper and evenly spread out by rolling a test tube on top. This set up was then overlaid with a thin non porous plastic adhesive film (Saran® wrap plastic) (Genetic Instrumentation Company) and evenly spread out again to eliminate any trapped air bubbles. The transparent covering gasket was placed evenly on top, the lid closed, and the vacuum turned on resulting in the formation of a vacuum seal. This was run for 1 hr and at the end of the cycle, the heating was turned off to let the gels cool before disrupting the vacuum seal, and thus prevent the gels from cracking.

6.10.4. Western Blot
6.10.4.1. Protein transfer

Immunoblotting after SDS-PAGE was performed according to the method of Towbin et al (1979). Prior to protein transfer, the nitrocellulose sheets (Biorad Laboratories., Hercules, USA), filter papers and pads were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol in distilled water, pH 8.3) for 10 min with gentle agitation. The polyacrylamide gels after SDS-PAGE electrophoresis were placed on the nitrocellulose membrane sandwiched between filter papers and scotbrite pads. Protein transfer was carried out using a Bio-rad transfer apparatus at 8° C using a constant current of 8 mA overnight, with the nitrocellulose membrane on the anodal side and gels on the cathode side.

Effectiveness of the transfer was evaluated by staining the gels after the transfer with Coomassie blue.
6.10.4.2. Immunostain technique

Immunostaining was performed by the method of Mutharia et al (1984), with slight modifications. After the protein transfer overnight, the membranes were blocked for 2 hr at room temperature with 3% bovine serum albumin (BSA) (Sigma Chemical Company) in assay buffer without Tween 20 (TBS) (50 mM Tris-HCl-buffer saline, pH 7.4). This was followed by three washes in assay buffer (50 mM-Tris-HCl, 0.05%-Tween 20, pH 7.4) (TBS-Tween 20) containing 1% BSA. The membranes were then incubated overnight at room temperature (23° C), with monoclonal anti-Lipid-A as primary antibody (8A1, Centorcor) diluted 1:500 in 1% BSA in assay buffer. This was followed by three 10 min washes in TBS-Tween and a further 1 hr incubation in secondary antibody, anti-mouse IgG horseradish peroxidase conjugate (Law Hospital, Lanark, UK.), at a dilution of 1:1000 in 1% BSA in TBS-Tween 20. After three 10 min washes as above, the membranes were incubated in freshly prepared chromogen solution. The chromogen was prepared by dissolving 20 mg of 3-amino-9-ethyl carbazole (AEC) (Sigma Chemical Company) in 2.5 ml dimethyl formamide, thoroughly mixing with 47.5 ml acetate buffer (50 mM sodium acetate buffer, pH 5.5), followed by filtration through Whatman No. 1 filter paper. Into the eluent was added 25 μl of hydrogen peroxide (30% (v/v)) (Sigma Chemical Company). The reaction was allowed to continue until the desired intensity of brown colour of the protein in the membranes was achieved. The reaction was terminated by washing the nitrocellulose membranes in distilled water followed by air-drying at room temperature.

To determine the cross reaction of secondary antibody to rat plasma proteins, rat serum was included as one of the samples and treated as above.
6.11. RESULTS

6.11.1. Endotoxin Activity

The protein content and endotoxin activity of four aliquots of trypanosome lysate and trypanosome enriched membrane proteins are presented in Tables 6.1 and 6.2, respectively. The protein content of the trypanosome lysates and the trypanosome enriched membrane proteins was 33.3 ± 4.0 mg/ml and 27.7 ± 4.1 mg/ml (mean ± SD) respectively. These gave LPS endotoxin activity equivalent of 59.4 ± 12.3 and 35.9 ± 6.7 EU/ml (mean ± SD) for the trypanosome lysate and trypanosome-enriched membranes respectively as measured by the LAL assay after serial dilution to fit the standard curve range.

6.11.2. Silver Stain

The sensitivity of the silver stain method for LPS following SDS PAGE was determined. This was done by taking a sample containing 6144 EU/ml of *E. coli* LPS, serially diluting it and using this as the starting material. The samples were treated and the gels stained as described in the materials and methods section. This showed that the silver stain was very sensitive and was able to detect as low as 24 EU/ml in the serially diluted *E. coli* LPS (Fig. 6.1).

During the running of the trypanosome samples, *E. coli* LPS and *S. minnesota* Lipid-A were included in each gel as the positive controls while distilled water was used as the negative control. Results are summarised in table 6.3. In all the gels stained with the silver stain, the lanes loaded with *E. coli* LPS (L) showed the classical ladder-like bands characteristic of smooth gram negative bacteria LPS. This was observed in samples before and after the PK digestion step. All the lanes loaded with endotoxin free water (W) and lipid-A (A) did not stain with the silver stain with or without the PK digestion step.

The silver stain for the trypanosome lysate is presented in figure 6.2a before PK digestion and figure 6.2 after PK digestion. The silver stain did not
stain the lanes loaded with trypanosome lysate (TL) before or after PK digestion.

To determine whether the lack of any staining in the trypanosome lysate samples was due to low concentration of endotoxin in the samples or loss of LPS during preparation of samples, whole trypanosome pellets were used as the starting material. This did not stain indicating that the pre analytical loss or concentration of LPS in samples was not a reason for the failure to stain these samples by the silver stain.

Similar to the trypanosome lysates, the trypanosome membranes (TM) did not stain by the silver stain before (Fig. 6.3a) or after (Fig. 6.3b) PK digestion. Attempts to increase the detection by the use of trypanosome membrane pellet as the starting material did not improve the staining.

6.11.3. Western Blot

To determine the presence of lipid-A in the trypanosome products, the Western blot technique using the monoclonal antibody 8A1 was used following SDS-PAGE and results are summarised in Table 6.4.

In each gel LPS and lipid-A were used as the positive controls while endotoxin free water was used as the negative control. In all the Western blots, monoclonal antibody 8A1 stained the lanes loaded with LPS (L) and lipid-A (A) as a single first migrating broad band of high mobility with the same molecular weight in both the LPS and lipid A. This was observed in the samples before and after PK digestion step.

The wells containing trypanosome lysates (TL) showed several slow migrating light staining bands only when loaded with samples without the PK digestion step (Fig. 6.4a). These bands were not detectable in any trypanosome lysate samples that were treated with PK (Fig. 6.4b) even when pellets from trypanosome lysate were used as the starting material.
The monoclonal antibody 8A1 stained several slow migrating bands in the lanes loaded with trypanosome membranes (TM), before the PK digestion step (Fig. 6.5a). The samples immunoblotted after PK digestion, did not indicate the presence of any bands even when trypanosome membranes pellet was used (Fig 6.5b).

To determine the crossreaction of the monoclonal antibody with plasma proteins in the trypanosome products, a serum sample from a normal rat was treated by the SDS-PAGE electrophoresis and immunostained as before. The protein sample did not stain with the monoclonal antibody indicating that the observed bands were not due to non-specific protein binding.

6.12. DISCUSSION

These results demonstrate that trypanosome lysate and membranes contain molecule(s) with endotoxin-like activity as measured by the LAL assay, although the endotoxin-like activity was low compared to the gram negative bacteria LPS.

After electrophoresis and staining, with a silver stain, the lanes containing trypanosome products and lipid-A, did not stain whereas the *E. coli* lipopolysaccharide used as the positive control, gave the classical ladder-like spread of different bands in the lanes loaded with LPS before and after the proteinase K digestion step. The distinctive ladder-like pattern is caused by the separation of a mixture of molecules of increasing molecular mass representing additional O-antigen units added to the basic lipid-A core oligosaccharide structure (Davies *et al*, 1991).

Sensitivity of the silver stain for LPS following SDS PAGE electrophoresis was high being able to detect LPS down to 24 EU/ml, and thus at the endotoxin levels detected by the LAL assay in the trypanosome products (> 26 EU/ml). LPS if present in the products should have been identified by the
silver stain. These findings indicate that the molecules(s) responsible for the endotoxin-like activity in trypanosomes are not of similar chemical structure to the gram negative bacteria LPS.

After Western blotting with the monoclonal antibody 8A1, the lipid-A and lipopolysaccharide stained as a single fast migrating broad band of similar size in both the PK digested and non PK digested samples. However, the trypanosome lysates and membranes lanes, showed lighter staining of several bands of higher molecular weight compared to that of lipid-A and LPS with the monoclonal antibody 8A1. This was only visible in the non PK digested trypanosome samples, whereas, in the PK digested samples, all these bands disappeared. This indicated that the molecule(s) being stained were destroyed by the PK treatment procedure suggesting that they were protein in nature or were associated with protein. Increasing the concentration of the PK treated trypanosome samples by using whole trypanosome and membrane pellets as the starting material did not improve the detectability, whereas bacterial LPS and lipid-A were both detectable at low concentration by the silver stain and Western blot staining techniques.

Detection of LAL activity in trypanosome lysate and membranes confirm that they contain endotoxin-like molecule(s) and likely to be the source of the increased levels of endotoxin-like activity in *T. b. brucei* infected mice demonstrated *in vivo* by Alafiatayo *et al* (1993) and in Chapter 5 of this thesis. Results from this study indicate the levels of activity were low compared to the LPS in *Trypanosoma cruzi* and gram negative bacteria LPS, confirming the observations of Pentreath, (1995).

The chemical nature of the molecule(s) responsible for the LAL activity in trypanosomes is not known. Although the LAL assay is activated by and is generally accepted as being specific for lipid-A, it has previously been shown to give positive reactions with non-endotoxin molecules including *Streptococcal*
exotoxin (Brunson and Watson, 1976), *Streptococcus faecalis* lipoteichoic acid (Fine, Kessler, Tabak and Shockman, 1977), and *Mycoplasma* lipoglycans (Seid, Smith, Guevarra, Hochstein and Barile, 1980; Weinberg *et al*., 1980). The LAL test has also detected the biological activity of endotoxin-like molecules purified from membranes of various organisms including *Chlamydia psittaci* (Lewis *et al*., 1979) *Plasmodium falciparum* (Jakobsen *et al*., 1988), and *Micropolyspora faeni* (Smith, Hill, Snyder and Burrel, 1978).

The finding that the monoclonal antibody 8A1 which is lipid-A specific (Mutharia *et al*., 1984; Bogard *et al*., 1987), reacted with trypanosome lysate without PK digestion suggests that trypanosomes contain molecule(s) with lipid-A or lipid-A-like epitopes. The visible bands on the immunoblots could not have been due to non-specific reaction of the monoclonal antibody with rat plasma proteins since the immunoblot treatment of normal rat serum did not give similar bands.

The molecule(s) giving the positive LAL and immunoblot reaction did not have a structure or chemical properties similar to gram negative bacteria lipopolysaccharide. The molecule(s) did not stain by the silver stain and also were unable to withstand PK digestion, unlike the *T. cruzi* LPS which has been shown to stain with the silver stain (de Simone *et al*., 1991), and also has been shown to be LPS (Ketteridge, 1978; Golberg *et al*., 1983). The possibility that the endotoxin was in very low amount, leading to decreased detectability, was unlikely since similar low levels of *E. coli* LPS were demonstrated on the gels by silver stain. The results of this study strongly suggest that endotoxin-like activity of *T. b. brucei* is not based on lipid-A and is therefore not of a similar chemical nature as bacterial lipopolysaccharide.

The observation that the trypanosome molecule(s), identified by the monoclonal antibody 8A1 by the Western blot, could not withstand PK digestion, suggests that the molecule(s) responsible for this activity could be
protein in nature or associated with protein. Indeed treatment of mice with variable surface glycoprotein (Cross, 1975), produces effects such as non-specific polyclonal B-cell activation, which are thought to be produced by an endotoxin molecule(s) released by trypanosomes (Greenwood, 1974).

Bacterial LPS, which is the most studied endotoxin, is known to be among the strongest inducers of cytokines (Mathison, Wolfson and Ulevitch, 1988). It is generally accepted that the inner core region of LPS is mainly responsible for the manifestation of endotoxicity, with lipid-A conveying the critical contribution (Kotani, Takada, Takahashi, Ogawa, Tsujimoto, Shimauchi, Ikeda, Okamura, Tamura, Harada, Tanaka, Shiba, Kusumoto and Shimamoto, 1986; Rietschel et al., 1987), and also being responsible for the adjuvant effects of endotoxin, including increased resistance to infection and cancer (Rietschel and Brade, 1992).

Cytokines are increasingly being recognised to be involved in the initiation of the pathological changes observed during parasitic infections (Rouzer and Cerami, 1980; Beutler and Cerami, 1987; Titus et al., 1991). During these parasitic infections, which include trypanosomiasis, increased serum cytokine levels have been demonstrated (Bancroft et al., 1983; Reincke et al., 1993).

Although the pathogenesis of African trypanosomiasis is yet to be fully understood, the production of cytokines during the infection is likely to be responsible for the production of some of the pathological changes observed, e.g. hypertriglyceridaemia (Rouzer and Cerami, 1980), and CNS inflammation (Hunter et al., 1991; Hunter et al., 1992a), common features of African trypanosomiasis. The mechanism of their production by the parasite is not well understood although the possible release of active trypanosome molecule(s) has been suggested and the endotoxin-like activity demonstrated in the present study, is likely to be involved in this process. Indeed in vitro trypanosome
products have been demonstrated to stimulate several cell types to release cytokines (Olsson et al, 1991; Gichuki, 1994), and similarly cells from infected hosts are known to show increased ability to release cytokines when stimulated (Sileghem et al, 1989; Sileghem et al, 1993). \textit{In vivo} administration of trypanosome products have also been shown to stimulate the release of active molecules including granulocyte-macrophage stimulating activity (Oka et al, 1989).

Among the molecules present in protozoan parasites such as trypanosomes, and known to be able to stimulate the production and release of cytokines is glycosyl phosphatidylinositol (Masterson, Raper, Doering, Hart and Englund, 1990; Bate and Kwiatkowski, 1994). The molecule is also found in trypanosomes but from this source was not found to stimulate cytokine production (Bate, Taverne, Roman, Moreno and Playfair, 1992; Jakobsen et al, 1995). Other phospholipids from malaria exoantigens have been shown to stimulate the production of TNF (Bate et al, 1992), these are also major constituents of the trypanosome plasma membrane (Dixon and Williamson, 1970; Rovis and Baekkeskov, 1980). The non lipid-A endotoxin-like activity demonstrated in trypanosomes in the present study, could be the same or similar to the molecule(s) responsible for the stimulation of cytokine release in the above publications.

The lack of a protective vaccine or safe non-toxic drugs for the treatment of trypanosomiasis, justify the search for detailed information concerning the interaction between the parasite and the host. The present work indicates that the characterisation of the trypanosome molecules is central to the understanding of the pathogenesis of African trypanosomiasis and thus may lead to the development of better disease management.

Further work that may lead to the understanding of the chemical nature of the molecule(s) responsible for the endotoxin-like activity in trypanosomes
should include the measurement of 2-keto-3-deoxy octulosonic acid (KDO) levels, a molecule found in most LPS linking the lipid-A and polysaccharide moieties via a ketosidic linkage. The cross reactivity of the monoclonal antibody 8A1 with non bacterial lipid-A could be confirmed by performing Western blots with *T. cruzi* LPS which has been characterised (Goldberg et al, 1983). Research could also be extended to analysis of other molecules purified from trypanosomes with biological activity such as the TLTF. Attempts should also be made to extract the endotoxin-like activity from purified trypanosomes by the standard techniques which have been applied in gram negative bacteria and *T. cruzi* and to then characterise the bioactive molecule(s).

6.13. CONCLUSION

This results suggest that trypanosomes contain molecule(s) with endotoxin-like activity. Although the molecule(s) responsible for this activity activate the proenzyme in the LAL assay as the lipid-A of gram negative bacteria, they are not chemically or structurally similar to the LPS of gram negative bacteria. These molecule(s) should be isolated and characterised as it is likely that they could play a central role in the pathogenesis of the disease process in African trypanosomiasis.
Table 6.1. The protein concentrations and endotoxin activity levels in *Trypanosoma brucei brucei* lysate.

<table>
<thead>
<tr>
<th>SAMPLES</th>
<th>PROTEIN CONCENTRATION (mg/ml)</th>
<th>ENDOTOXIN ACTIVITY (EU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33.4</td>
<td>65.3</td>
</tr>
<tr>
<td>2</td>
<td>38.2</td>
<td>46.8</td>
</tr>
<tr>
<td>3</td>
<td>28.4</td>
<td>73.6</td>
</tr>
<tr>
<td>4</td>
<td>33.3</td>
<td>51.9</td>
</tr>
<tr>
<td>MEAN ± SD</td>
<td>33.3 ± 4.0</td>
<td>59.4 ± 12.3</td>
</tr>
<tr>
<td>SAMPLES</td>
<td>PROTEIN CONCENTRATION (mg/ml)</td>
<td>ENDOTOXIN ACTIVITY (EU/ml)</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>1</td>
<td>25.2</td>
<td>35.1</td>
</tr>
<tr>
<td>2</td>
<td>33.1</td>
<td>38.9</td>
</tr>
<tr>
<td>3</td>
<td>23.9</td>
<td>26.9</td>
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<tr>
<td>4</td>
<td>28.4</td>
<td>42.6</td>
</tr>
<tr>
<td>MEAN ± SD</td>
<td>27.7 ± 4.1</td>
<td>35.9 ± 6.7</td>
</tr>
</tbody>
</table>

**Table 6.2.** The protein concentrations and endotoxin activity levels in *Trypanosoma brucei brucei* membranes.
Fig. 6.1. Silver stain SDS-PAGE gel loaded with serially diluted *E. coli* LPS. The arrow indicates the direction of decreasing concentration of LPS. A-highest concentration and B- lowest concentration.
Fig. 6.2. Silver stain SDS-PAGE gel loaded with LPS (L), Lipid-A (A), saline (S) and trypanosome lysate (TL). Figure 6.2a samples were not treated with PK prior to electrophoresis while in Figure 6.2b the samples were treated with 25 μg of PK for 60 min. Note the ladder-like staining of the *E. coli* LPS.
**Fig. 6.3** Silver stain SDS-PAGE gel loaded with LPS (L), Lipid-A (A), saline (S) and membrane-enriched trypanosome fraction (TM). Fig. 6.3a samples were not treated with PK prior to electrophoresis while in Fig. 6.3b the samples were treated with 25 μg of PK for 60 min. Note the classical ladder-like staining of bacterial LPS.
Table 6.3. The summary of the silver stained SDS PAGE gels following electrophoresis of bacterial and trypanosome products. N-No visible bands; + Single band; ++-More than one band.

<table>
<thead>
<tr>
<th>SAMPLES</th>
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<th>AFTER PK DIGESTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>LPS</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Lipid-A</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Trypanosome membranes</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Trypanosome lysate</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>
Fig. 6.4. Western blot analysis of LPS (L), lipid-A (A), saline (S) and trypanosome lysate (TL). Figure 6.4a the samples were not treated with PK before electrophoresis while Figure 6.4b the samples were pre-treated with 25 μg of PK for 60 min before electrophoresis. Note the staining of *E. coli* LPS and lipid-A from *S. minnesota* with or without PK digestion, and trypanosome lysate without PK digestion.
**Fig. 6.5.** Western blot analysis of LPS (L), lipid-A (A), saline (s) and membrane enriched trypanosome fractions (TM). Figure 6.5a the samples were not treated with PK while in Figure 6.5b the samples were pre-treated with 25 μg of PK before electrophoresis. Note the staining of LPS and lipid-A with or without PK digestion and trypanosome enriched membranes without PK digestion.
Table 6.4. The summary of the Western blot from SDS PAGE gels following electrophoresis and transfer of bacterial and trypanosome products. N-No visible band; +-Single band; ++-More than one band.

<table>
<thead>
<tr>
<th>SAMPLES</th>
<th>BEFORE PK DIGESTION</th>
<th>AFTER PK DIGESTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>LPS</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lipid-A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trypanosome membranes</td>
<td>++</td>
<td>N</td>
</tr>
<tr>
<td>Trypanosome lysate</td>
<td>++</td>
<td>N</td>
</tr>
</tbody>
</table>
CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION
This thesis is a study on the pathogenesis of African trypanosomiasis using mice infected with either *Trypanosoma brucei brucei* or *T. congolense*, with particular reference to the acute phase response (APR). Previous studies have shown that trypanosome infections cause pathological changes in a wide range of organs and tissues accompanied by alterations of biochemical levels of different blood components. However, only a few studies have been carried out on the APR in trypanosomiasis (Thomasson *et al.*, 1973; Cook, 1979; Esievo *et al.*, 1984; Pluschke *et al.*, 1986; Ndungu *et al.*, 1991; Shapiro and Black, 1992), and little is known about the possible pathophysiological role of acute phase proteins (APP) during the disease process (Shapiro and Black, 1992).

The working hypothesis of the present investigations is that trypanosomes contain and release an endotoxin-like molecule(s) which, through the over-stimulation of endogenous cytokines, leads to the production of the observed pathological changes such as the APR.

The objectives of this investigation were to develop a direct ELISA for measurement of serum amyloid P-component (SAP), a major murine APP, assess the APR during a tissue invasive and non-invasive trypanosome infection, and to identify the origin of the endotoxin-like molecule(s) released during the course of a trypanosome infection.

Chapter 3 describes the development of the ELISA for the estimation of SAP. The advantage of this assay was that it utilised only small amounts of test plasma making it possible to assay SAP serially in small animals such as the mouse. The assay was also sparing in the use of antigen and antibodies.

This ELISA was used to measure the plasma levels of SAP in normal and trypanosome-infected mice (Chapters 4 and 5). The results obtained showed that both *T. b. brucei* (invasive) and *T. congolense* (non-invasive) infections in mice, induced an increase in the plasma levels of the APP,
haptoglobin and SAP (Chapter 4). This response was accompanied by cellular infiltration and damage to the liver, spleen and the brain in the *T. b. brucei* infections. In *T. b. brucei* infections, both APP increased rapidly reaching peak levels within 10 DAI; thereafter SAP decreased to about baseline levels, while Hp remained elevated at 50% peak levels. Similarly, following *T. congolense* infections, both APP increased and reached peak levels early in the infection. Thereafter, SAP decreased but then increased around 30 DAI before starting to fall. Haptoglobin, on the other hand, increased rapidly to peak levels, decreased by a 50% and thereafter remained at this elevated level.

The finding that both invasive and non-invasive trypanosome infections resulted in increased plasma levels of APP, indicated that the trypanosomes do not induce the release of cytokines and APP, solely through the invasion and damage of tissue. Indeed, in *T. b. brucei* infections, APP started to increase before trypanosomes could be demonstrated in circulation and peaked before any severe tissue damage had occurred; later in the infection, severe pathology was concomitant with a decrease of 50% in haptoglobin and a near baseline concentration of SAP.

Following treatment of *T. congolense*-infected mice with trypanocidal drug diminazine aceturate, which is curative in this infection, haptoglobin concentrations decreased rapidly. In contrast in *T. b. brucei*-infected mice in which the treatment is subcurative with trypanosomes persisting in the brain, haptoglobin concentrations remained elevated, indicating that the trypanosomes and/or the pathology in the CNS of the mice might still be playing a part in the continuing stimulation of the hepatic APR. This observation needs to be further investigated in a more extensive study with follow-up of the animals for a longer period after a curative and subcurative treatment.

Acute phase proteins reflect a complex cytokine reaction but remain in the circulation longer than cytokines. Acute phase proteins therefore, provide an
indicator of cytokine activity during the disease process. Indeed in mice, a strong correlation between the ability to produce IL-1 and the SAP response has been demonstrated (Mortensen et al, 1983).

Although increased endotoxin-like activity has been previously demonstrated in plasma of *T. b. brucei*-infected mice, and trypanosomes are documented to contain molecules with endotoxin-like activity, the source and pathological significance of this serum endotoxin-like activity during trypanosome infection has not yet been elucidated. Chapter 5 describes a series of studies on mice infected with *T. b. brucei* and kept under an umbrella cover with the antibiotics norfloxacin, a systemic broad spectrum antibiotic, or polymyxin-B an antibiotic that binds to and inactivates LPS. The levels of plasma endotoxin-like activity remained elevated and were not affected by treatment with either antibiotics. This finding suggests that the increased endotoxin-like activity is of parasite origin. Similarly, pathological changes assessed by APP, Hp and SAP, and by histological examination of tissues, were not affected significantly by the antibiotic treatments. In one study a slight decrease in the Hp concentration occurred in the norfloxacin-treated mice during late infection, and a decrease in the cellular infiltration of the liver was observed in norfloxacin-treated mice during the early stages of infection. These results suggest that although secondary bacterial infections might influence the pathological changes during trypanosomiasis, the trypanosome parasites are likely to be responsible for the initiation of the pathological changes observed during infection in mice.

The study also attempted to characterise the chemical nature of the molecule(s) responsible for endotoxin-like activity in trypanosomes (Chapter 6). The lipid-A moiety of gram negative bacteria LPS is responsible for the pathogenic effects of LPS and in activating the LAL assay. Trypanosomes were therefore analysed for the presence of LPS by a silver stain that preferentially
stains LPS, and for the lipid-A moiety by Western blotting with a monoclonal antibody. Results suggested that the parasites contain molecule(s) with endotoxin-like activity and might have epitopes similar to lipid-A, but are in structure different from the LPS of gram negative bacteria. Further studies need to be undertaken to purify and characterise the molecules with endotoxin-like activity and compare with other non-bacterial LPS molecules of known chemical structure such as *T. cruzi* a close relative of the African trypanosome which is reported to contain an LPS structure (Goldberg et al, 1983).

These studies have shown that the African trypanosomes *T. b. brucei* and *T. congolense*, induce an APR in mice. As the mouse model is an excellent reflection of the disease in man and the major animal species, it is probable that the response also occurs in these hosts. The APR is associated with an increase in plasma endotoxin-like activity and APP. These results have several clinical implications in trypanosomiasis.

The APP might be used as markers in different aspects of trypanosomiasis and in examining the effect of trypanocidal and non-trypanocidal drugs on the host during an active infection. With the emerging evidence that some of the APP might be playing an active role in the host response, further studies need to be conducted to ascertain this possibility. For example, haptoglobin at acute phase concentrations is known to be immunosuppressive, whereas, $\alpha_2$-macroglobulin is known to inhibit the proliferative response of lymphocytes and also the antibody-dependent cell-mediated cytotoxicity and natural killer cell activity. Serum amyloid-P has been shown to augment *in vitro* listericidal activity of macrophages and thus might act as a mediator of the increased phagocytic activity observed during trypanosomiasis. C-reactive protein and SAP are known to induce cytokine production from monocytes/macrophages, while $\alpha_2$-macroglobulin binds to IL-6 and IL-1, an effect that protects IL-6 from degradation by proteases and thus
could prolong its half-life and possibly its effect on the host. These acute phase proteins might thus be involved in the production of the cytokines and also affect their plasma half-life and thus could influence the course of the disease. Haptoglobin in circulation binds free haemoglobin released during haemolysis, thus preventing glomerular filtration of haemoglobin which could lead to kidney damage and irreversible loss of iron from the body. Thus this action might conserve iron during haemolytic anaemia and reduce the severity of anaemia during infections.

In conclusion, this study has provided evidence that the induction of the disease process in African trypanosomiasis might be the result of an endotoxin-like cytokine-inducing molecule(s) present in the trypanosome, a molecule(s) with similar activity to the LPS found in gram negative bacteria.
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APPENDICES
APPENDIX 1:

Stock Solutions for SDS-PAGE Buffers and Gel Mixes

Stock Acrylamide (Monomer solution-30%, 2.7% Bis)
Acrylamide 29.2 g  
Methylene-bisacrylamide 0.8 g  
Distilled water 100 ml  

Separating Gel Buffer (1.6 M Tris-HCL pH 8.8)
Tris-HCL 18.15 g  
Distilled water 100 ml  

Stacking Gel Buffer (0.5 M Tris-HCL pH 6.8)
Tris-HCL 3.0 g  
Distilled water 50 ml  

10% SDS
SDS 50 g  
Distilled water 500 ml  

Initiator (10% ammonium persulphate)
Ammonium persulphate 0.5 g  
Distilled- water 5 ml  

Resolving Gel Overlay (0.375 M Tris-HCL pH 8.8, 0.1% SDS)
Resolving gel buffer 25 ml
10% SDS 1 ml
Distilled water 74 ml

15% SDS-PAGE Separating Gel
Stock acrylamide 15 ml
1.6 M Tris-HCL (pH 8.8) 7.5 ml
10% SDS 300 µl
Distilled water 7 ml
Ammonium persulphate 150 µl
TEMED 15 µl

4% Stacking Gel
Stock acrylamide 1.34 ml
0.5 M Tris-HCL (pH 6.8) 2.5 ml
Distilled water 6 ml
10% SDS 100 µl
TEMED 15 µl

Protein Treatment Buffer (Sample buffer)
2.5% SDS
2.5% dithreioerythriol
0.1 M Tris-HCL (pH 6.8)
10% glycerol
0.01% bromophenol blue
APPENDIX 2:

LPS Treatment Buffer

Treatment Buffer (Sample buffer)

2.5% SDS
2.5% dithreioerythriol
0.1 M Tris-HCL (pH 6.8)
10% glycerol
40 mM EDTA
0.01% bromophenol blue

Electrophoresis Tank Buffer

SDS-PAGE Tank Buffer (0.025 M Tris, pH 8.3, 0.19M glycine, 0.1% SDS)

Glycine 14.4 g
Tris base 3.1 g
SDS 1.0 g
Distilled water 1 L
APPENDIX 3:

Coomassie Blue Staining and Destaining Solutions

Coomassie Blue Staining Solution
Coomassie blue 0.5 g
Methanol 300 ml
Glacial acetic acid 100 ml
Distilled water-to 1 L

Destaining Solution
Methanol 300 ml
Glacial acetic acid 100 ml
Distilled water to 1 L
APPENDIX 4:

Western Blot Buffers

Transfer Buffer (25 mM Tris, 192 mM glycine, 5 % methanol, pH 8.3)

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight/Volume</th>
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<tbody>
<tr>
<td>Tris-HCL</td>
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<tr>
<td>Glycine</td>
<td>28.33 g</td>
</tr>
<tr>
<td>20 % Methanol</td>
<td>0.5 L</td>
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<tr>
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Western Blot Assay Buffer (20 mM Tris-HCL, 150 mM NaCl, 0.05 % Tween-20, pH 7.4)

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<td>Sodium chloride</td>
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<td>Tween-20</td>
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</table>
ALWAYS REMEMBER THAT THE FEAR OF THE LORD IS THE BEGINNING OF WISDOM
PSALM 111: 10.