

**THE ROLE OF ASTROCYTES IN THE NEUROPATHOGENESIS OF AFRICAN  
TRYPANOSOMIASIS**

by

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## **DECLARATION**

I hereby declare that the work presented in this thesis is original and was conducted solely by the author, except where collaboration with others is acknowledged.

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**DEDICATION**

**TO MY PARENTS**

**AND**

**MY SON**

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## **ABBREVIATIONS**

<b>ALT</b>	Alanine transferase
<b>AMP</b>	Adenosine monophosphate
<b>cAMP</b>	Cyclic adenosine monophosphate
<b>AP</b>	Alkaline phosphatase
<b>ATP</b>	Adenosine triphosphate
<b>BAL</b>	British anti-Lewisite
<b>BBB</b>	Blood-brain barrier
<b>BSA</b>	Bovine serum albumin
<b>°C</b>	Degrees celcius
<b>Ca<sup>++</sup></b>	Calcium ions
<b>CNS</b>	Central nervous system
<b>CSF</b>	Cerebrospinal fluid
<b>CSFs</b>	Colony stimulating factors
<b>CSF-1</b>	Colony stimulating factor 1
<b>DAB</b>	Diaminobenzidine
<b>DEAE</b>	Diethylaminoethyl
<b>DMEM</b>	Dulbeccos modified Eagles medium
<b>DMF</b>	Dimethylformamide
<b>DNA</b>	Deoxyribonucleic acid
<b>cDNA</b>	Complementary deoxyribonucleic acid
<b>DNase</b>	Deoxyribonuclease
<b>DFMO</b>	Difluoromethylornithine
<b>ECs</b>	Endothelial cells
<b>EDTA</b>	Ethylene diamine tetraacetic acid
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>ELAM-1</b>	Endothelial leukocyte adhesion molecule 1
<b>FCS</b>	Fetal calf serum

<b>g</b>	<b>Gram</b>
<b>g</b>	<b>Relative centrifugal force</b>
<b>GFAP</b>	<b>Glial fibrillary acidic protein</b>
<b>G-CSF</b>	<b>Granulocyte colony stimulating factor</b>
<b>GM-CSF</b>	<b>Granulocyte-macrophage colony stimulating factor</b>
<b>GPF</b>	<b>Glial promoting factor</b>
<b>GSF</b>	<b>Glial cell stimulating factor</b>
<b>HBSS</b>	<b>Hanks balanced salt solution</b>
<b>HCl</b>	<b>Hydrochloric acid</b>
<b>ICAM-1</b>	<b>Intercellular adhesion molecule 1</b>
<b>IFN</b>	<b>Interferon</b>
<b>Ig</b>	<b>Immunoglobulin</b>
<b>IgG</b>	<b>Immunoglobulin G</b>
<b>IgM</b>	<b>Immunoglobulin M</b>
<b>IL-1 to 6</b>	<b>Interleukin 1 to 6</b>
<b>IMP</b>	<b>Inosine monophosphate</b>
<b>I.U.</b>	<b>International units</b>
<b>K<sup>+</sup></b>	<b>Potassium ions</b>
<b>Kg</b>	<b>Kilogram</b>
<b>l</b>	<b>Litre</b>
<b>L15</b>	<b>Leibovitch medium</b>
<b>LDL</b>	<b>Low density lipid</b>
<b>LFA</b>	<b>Leukocyte function activating factor</b>
<b>LPS</b>	<b>Lipopolysaccharide</b>
<b>M</b>	<b>Molar concentration</b>
<b>mA</b>	<b>Milliampere</b>
<b>mg</b>	<b>Milligram</b>
<b>ml</b>	<b>Millilitre</b>

mM	Millimolar
Mg <sup>++</sup>	Magnesium ions
MHC	Major histocompatibility complex
MIP-1	Macrophage inflammatory protein 1
MNTI	Methylnitroimidazole
6-MP	6-mercaptopurine
N	Normal concentrations
Na <sup>+</sup>	Sodium ions
NH <sub>4</sub> <sup>+</sup>	Ammonium ions
NGS	Normal goat serum
dNTPs	Deoxynucleoside triphosphates
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PG	Prostaglandin
PGD	Prostaglandin D
PGE	Prostaglandin E
PLL	Poly-L-lysine
PSG	Phosphate buffered saline with glucose
PTRE	Post-treatment reactive encephalitis
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
SBTI	Soya bean trypsin inhibitor
SH	Sulphydryl
TBE	Tris boric EDTA buffer
TBS	Tris buffered saline
T <sub>H</sub> 1	Type 1 T-helper cells

$T_H2$	Type 2 T-helper cells
TMB	Tetramethyl benzidine
TNF	Tumor necrosis factor
Tris	Tris(hydroxymethyl)methylamine
VCAM-1	Vascular cell adhesion molecule 1
VLDL	Very low density lipids
WHO	World Health Organisation
XMP	Xanthine monophosphate
$\alpha$	Alpha
$\beta$	Beta
$\gamma$	Gamma
$\mu\text{g}$	Micrograms
$\mu\text{m}$	Micrometers
$\mu\text{l}$	Microlitres

## SUMMARY

This thesis concerns the role of astrocytes in the neuropathogenesis of trypanosomiasis caused by *Trypanosoma brucei brucei* in mice, a model of the human disease caused by *T.b. rhodesiense* and *T.b. gambiense*.

Chapter 1 of this thesis includes a literature review of the published work on human African trypanosomiasis (HAT) with a bias on the pathology and possible pathogenic mechanisms, particularly as they relate to the central nervous system (CNS). It also reviews the role of astrocytes in the normal and diseased CNS, and their possible role in the genesis of CNS pathological changes during trypanosome infections. The biological activities of cytokines reported to be produced in the CNS of trypanosome-infected animal models are considered. The chronic trypanosomiasis mouse model, used in this study, is described highlighting the similarities of the disease syndromes in this model to HAT.

Chapter 2 describes the experimental techniques used in this study.

Chapter 3 demonstrates the occurrence of astrocyte activation, as judged by an increase in the intensity of glial fibrillary acidic protein (GFAP) staining and morphological changes, within the CNS of mice chronically infected with *T.b. brucei*. It was shown that astrocyte activation first occurred 21 days after infection around the ventricles and the choroid plexus, along the choroid fissure, including, the hippocampus and the base of the cerebellum. It was also demonstrated that astrocyte activation preceded the infiltration of the choroid fissure, the perivascular spaces and the meninges, with inflammatory cells. It would appear that this activation involves production of mediators of inflammation which initiate extravasation of inflammatory cells, and possibly induce further astrocyte activation. From these initial sites, astrocyte activation spreads first to the white matter, the corpus callosum and the cerebellar lobes, and then to the grey matter, of both the cerebrum and the cerebellum. The infiltration of inflammatory cells seemed to enhance the spread and the degree

of astrocyte activation. It is known that these areas where initial astrocyte activation occurred, have an incomplete blood-brain barrier and, they have been reported as the first sites of trypanosome invasion, at around day 14 of infection. This suggested that the astrocyte activation observed in these areas, was the first CNS response to invasion by trypanosomes. Subcurative therapy with Berenil, seemed to facilitate inflammatory cell infiltration and astrocyte activation and to hasten the progress of the CNS pathological changes. Treatment of relapsed infections with a second subcurative dose of Berenil increased the inflammatory cell infiltration and the astrocyte activation, resulting in a post-treatment reactive encephalitis (PTRE), similar to that observed in human reactive arsenical encephalopathy (RAE). Curative trypanocidal therapy rapidly removed the infiltrating cells followed by a slow regression of astrocyte activation. It was concluded that the CNS pathological changes are the host's response to invasion by the parasite and that astrocytes played an important role in initiating these changes, possibly by producing inflammatory mediators such as cytokines.

Chapter 4 demonstrates the response of cultured astrocytes, as judged by cytokine transcript expression, to *in vitro* stimulation with trypanosomes, whole trypanosome lysate and variable surface glycoprotein (VSG). Using reverse transcription, cDNA amplification by polymerase chain reaction, and gel electrophoresis, it was found that astrocytes responded by expressing transcripts for the cytokines IL-1 $\alpha$  and - $\beta$ , IL-6, TNF $\alpha$ , MIP-1 and GM-CSF. Trypanosomes and whole trypanosome lysate were more potent stimuli than VSG in inducing expression of cytokine transcripts. To investigate translation of the gene transcripts into cytokine protein, immunocytochemistry using anti-mouse cytokine antibodies, was performed on astrocytes stimulated with trypanosome lysate *in vitro*. Cytokine protein for IL-1 $\alpha$ , TNF $\alpha$  and IL-6, was detectable by 2 hours after exposure to trypanosome lysate. The cytokines

shown to be expressed, in this study, are known to act in tandem and synergy to perform important inflammatory functions, including, leukocyte extravasation, migration, proliferation, adhesion-molecule and MHC-antigen expression, and cytokine production by inflammatory cells, microglia and endothelial cells. In addition, the same cytokines activate astrocytes in an autocrine manner, causing proliferation, MHC-antigen and adhesion-molecule expression. It is proposed that astrocytes respond to trypanosome invasion of the CNS by producing cytokines which recruit inflammatory cells into the subarachnoid and perivascular spaces, and the choroid fissure. The recruited inflammatory cells respond to these cytokines by proliferating, differentiating to antibody-producing plasma cells or to actively phagocytic cells, in the case of macrophages, as well as by producing cytokines. Autocrine and paracrine stimulation of the intrinsic brain cells and the infiltrating inflammatory cells, progressively aggravate the CNS lesions, leading to severe meningoencephalitis and later neuronal degeneration, characteristic features of both advanced human and experimental trypanosomiasis.

With the identification of astrocytes as one source of key inflammatory cytokines within the CNS of *T.b. brucei*-infected mice, this study explores how drugs known to prevent and attenuate the Berenil-induced PTRE, affects astrocyte activation. Eflornithine and azathioprine are such drugs.

Chapter 5 investigates the effect of eflornithine, a reversible inhibitor of ornithine decarboxylase (ODC), on astrocyte activation in mice infected with the eflornithine-resistant trypanosomes (*T.b. brucei* GVR 35/C1.3 DFMO 5), and on cytokine production by cultured astrocytes stimulated with lipopolysaccharide (LPS) *in vitro*. Eflornithine was administered as a 2% solution in drinking water *ad libitum* for 7 or 14 days. When administered from day 21 of infection, it delayed the onset of astrocyte activation and the inflammatory cell infiltration into the CNS. To investigate the effect of eflornithine on the induction of PTRE, Berenil treatment was administered in such away that that it was given at the

beginning or the end of an eflornithine course, or from 7 days after the start of treatment with eflornithine. It was found that eflornithine prevented the astrocyte activation and infiltration of inflammatory cells, for as long as it was administered. Inflammatory cell infiltration was detectable 15 days after discontinuation of eflornithine therapy. To investigate the effect of eflornithine on an established PTRE, eflornithine therapy was started 7 days after the first or the second Berenil treatment. In each case, it was found that eflornithine ameliorated both the inflammatory-cell infiltration and astrocyte activation. To examine the effect on cultured astrocytes, eflornithine was introduced to cultured astrocytes simultaneously with 10 $\mu$ g/ml LPS. Eflornithine was used at two concentrations: 0.5mM, reflecting concentrations reaching the CNS of HAT patients during a therapeutic eflornithine regimen, and 5mM, the usual concentration applied to *in vitro* experiments. At therapeutic doses, eflornithine inhibited the LPS-induced expression of IL-1 $\alpha$ . At higher (experimental) dose, it inhibited the expression of IL-1 $\alpha$ , TNF $\alpha$  and IL-6, reduced expression of IL-1 $\beta$  to baseline levels, shortened the duration of GM-CSF expression from 8 to 4 hours, but did not affect the expression of MIP-1. It is proposed that, by inhibiting ODC, eflornithine prevents the proliferation of microglia and inflammatory cells, and by inhibiting IL-1 production, it prevents the cascade of events that leads to the expression of adhesion molecules on endothelial cells, leukocyte extravasation and proliferation.

Chapter 6 examined the effect of azathioprine, a non-steroidal anti-inflammatory drug, on astrocyte activation *in vivo* and *in vitro*. To investigate the effect on the induction of PTRE, azathioprine was administered at 100mg/kg intraperitoneally (i.p.) for 7 or 14 days, starting from day 24, followed by Berenil treatment at 40mg/kg i.p. on day 26 after infection of mice with *T.b.brucei* GVR 35/C1.5. It was found that azathioprine completely prevented the inflammatory cell infiltration but did not prevent astrocyte activation. To investigate the effect of azathioprine on an established PTRE,

azathioprine was administered 7 days after the first Berenil treatment, or at the time of the second Berenil treatment. It was found that when given 7 days after Berenil therapy, azathioprine did not reverse the astrocyte activation or the inflammatory cell infiltration, and that when given at the time of the second Berenil treatment it stopped the inflammatory cell infiltration but had no effect on astrocyte activation. It was concluded that azathioprine therapy at the time of trypanocidal therapy might be beneficial since administration at the time of Berenil treatment (even after relapse of parasitaemia) seemed to slow down the progress of the PTRE. It is possible that azathioprine therapy might arrest the development of clinical reactive arsenical encephalopathy (RAE) especially when used in combination with the first-time trypanocidal treatment.

The results from chapter 5 and 6 indicate that both eflornithine and azathioprine, when used in conjunction with trypanocidal therapy might reduce the incidence of RAE. The results show that eflornithine is superior to azathioprine in this respect. As an ODC inhibitor, it is possible that eflornithine inhibits proliferation of both the infiltrating inflammatory cells and the activated astrocytes, and by blocking both the ODC and the cytokine production by astrocytes and, possibly, by the infiltrating inflammatory cells, the cascade of events that leads to meningeal inflammation. It would appear that using a drug aimed at a specific mediator would be more effective in preventing inflammatory reactions in the brain than using a cell-type specific inhibitor.

## **CHAPTER 1**

### **A GENERAL OVERVIEW OF HUMAN AFRICAN TRYPANOSOMIASIS**

## 1.1 HISTORICAL PERSPECTIVE AND IMPORTANCE

Foci of human sleeping sickness have existed for a long time in Africa to the extent that the disease and its transmission has passed into folklore (Service, 1978). As the Arabs and the Portuguese explorers developed trade between Africa and the outside world, well-documented accounts on the clinical signs of both human and animal trypanosomiasis were reported and in the latter case, its association with the tsetse fly was documented (reviewed by Lambrecht, 1964). Much later in the nineteenth century settlers and hunters in Southern Africa and explorers in Central and Eastern Africa observed the association between the tsetse fly and a disease that killed their horses and cattle. Tsetse in the Setswana language of Botswana means "a fly destructive of cattle". Fuller (1924) attributed many of the hardships that the early European settlers encountered in Africa as being due to animal trypanosomiasis. As European settlers and hunters moved Northwards across the Vaal River into the Transvaal of South Africa, they began to encounter a disease of cattle and horses referred to as 'nagana' in the Zulu language.

Shortly after the British annexed Zululand in 1887, game laws were enforced that protected wild game from the hunt by indigenous people. The result was an increase in the number of game animals followed by a corresponding increase in the cases of animal trypanosomiasis. In 1894, David Bruce was assigned to investigate the cause of numerous deaths in cattle and horses. In 1895, he and his wife, Mary, discovered trypanosomes in the blood of infected animals and concluded that trypanosomes were the causative agents of the disease 'nagana' (Bruce, 1895). He investigated and showed that tsetse flies transmitted trypanosomes and that wild animals could serve as disease reservoir hosts for the infection (Bruce, 1897). In 1909, Klein showed that trypanosomes underwent cyclical development in tsetse flies, thus proving they were the biological vectors of the disease.

In 1886, Bruce sent a trypanosome-infected dog to England for further

investigation. Plimmer and Bradford (1899) isolated the causative parasite from the blood of that dog and named it *Trypanosoma brucei* in honour of Dr Bruce. Shortly thereafter, some of the most important trypanosomes affecting domestic animals in Africa such as, *T.congolense* (Brodin, 1904), *T.suis* (Ochman, 1905) and *T.simiae* (Bruce, Hamerton, Bateman, Mackie and Bruce, 1911) were identified and their life cycles determined.

The causative organism of human sleeping sickness was isolated from a patient from Gambia by Forde in 1901, and was first recognised as a trypanosome by Dutton (1902) who named it *T. gambiense* (reviewed by Davies, 1962). In 1902, Castellani had independently isolated the same causative agent of sleeping sickness from the cerebrospinal fluid of a patient in Uganda and named it *T.ugandense* (Castellani, 1903; Davies, 1962). The name *T.rhodesiense* was given by Stephens and Fantham (1910) to a strain isolated from a sleeping sickness patient from Rhodesia, which was distinguished from *T.gambiense* by the presence of posteronuclear forms and by its greater virulence for laboratory rodents.

Two forms of human African trypanosomiasis are now recognised. The chronic form caused by *T.b. gambiense* and the acute form caused by *T.b. rhodesiense*. Both forms of the disease are invariably fatal in the absence of chemotherapy. Recent estimates suggest that 50 million people are at risk of infection in Africa (Kuzoe, 1989) and the reported annual incidence of 20,000 to 25,000 new cases may be a gross underestimate owing to the rural location of this disease. Indeed, the World Health Organisation believes that these figures are more likely to be between 200,000 and 300,000 new cases annually (Kuzoe, 1993a). The present Ugandan epidemic caused by the acute *rhodesiense* form of the disease and the outbreaks in the Southern Sudan, since the 1970s, of the chronic *gambiense* form illustrate the profound effects of the breakdown of diagnostic and surveillance facilities brought about through civil unrest

(Goodwin, 1985).

The diagnosis of trypanosomiasis is based on detection of parasites in the blood, the cerebrospinal fluid (CSF) and the lymph node aspirates from patients (WHO, 1986). Parasites, especially in the *gambiense* form of the disease, are not always detectable in patients due to the intermittent nature of parasitaemia and the tissue invasive nature of the parasites. Immunological methods of diagnosis, based on the detection of antibodies to the parasites and the detection of parasite antigen in the sera and CSF of patients are also used (Luckins, Gray and Rae, 1978; Nantulya, 1989). However, they show long persistence after curative regimens. This makes it difficult to diagnose ongoing infections for treatment. More accurate methods, using molecular techniques for the detection of parasite DNA, have been developed but are very expensive and impractical in the very rural areas of Africa where trypanosomiasis occurs (WHO, 1986).

There is no satisfactory treatment for trypanosomiasis largely because the available medicaments can be toxic and are uncertain in action. Moreover, there are reports of resistance in some trypanosome strains to Pentamidine and Melarsoprol, the drugs commonly used for treatment of human trypanosomiasis, (Williamson, 1970; Kayembe and Wery, 1972; Oganda, 1974; Bacchi, Nathan, Livingston, Valladares, Saric, Sayer, Njogu and Clarkson, 1990) and cross resistance to these drugs was reported as early as 1951 (Rollo and Williamson, 1951). Nor has there been much enthusiasm in the pharmaceutical industry to develop new drugs. The research is difficult and unpredictable; modern requirements for toxicology before clinical trials are lengthy and very expensive; new discoveries are open to piracy by competitors; and the people who need the drugs are mostly too poor to pay for them (Kuzoe, 1993b).

No vaccine is available because of the formidable problem of antigenic variation. Individual clones of trypanosomes have been reported to develop at least 100 different variable antigenic types (Capbern, Giroud, Baltz and Mattern, 1977), and genetic analysis suggests that between 300 and 1000

different serotypes could be produced (Van der Ploeg, Valerio, De Lange, Bernards, Borst and Grosveld, 1982). Immunocompetent hosts produce lytic and opsonising antibodies directed against the surface antigens of the trypanosomes. These antibodies can clear parasitaemia, but cannot eliminate the trypanosome infection because of the continuous appearance of new variants (Turner, 1985).

Although serious efforts have been made to reduce transmission of the disease, complete eradication of trypanosome vectors has not proved possible due to: political and civil unrest, worsening economies of endemic countries and competing health priorities. As a result, the disease has not been given due attention by the national health authorities, leading to recrudescence of old foci and geographical spread (Kuzoe, 1993b). Furthermore, sleeping sickness foci and epidemics can extend across country borders with difficult relations between neighbouring countries hindering intercountry cooperation (Kuzoe, 1993b). Consequently, trypanosomiasis has remained a major concern in endemic countries .

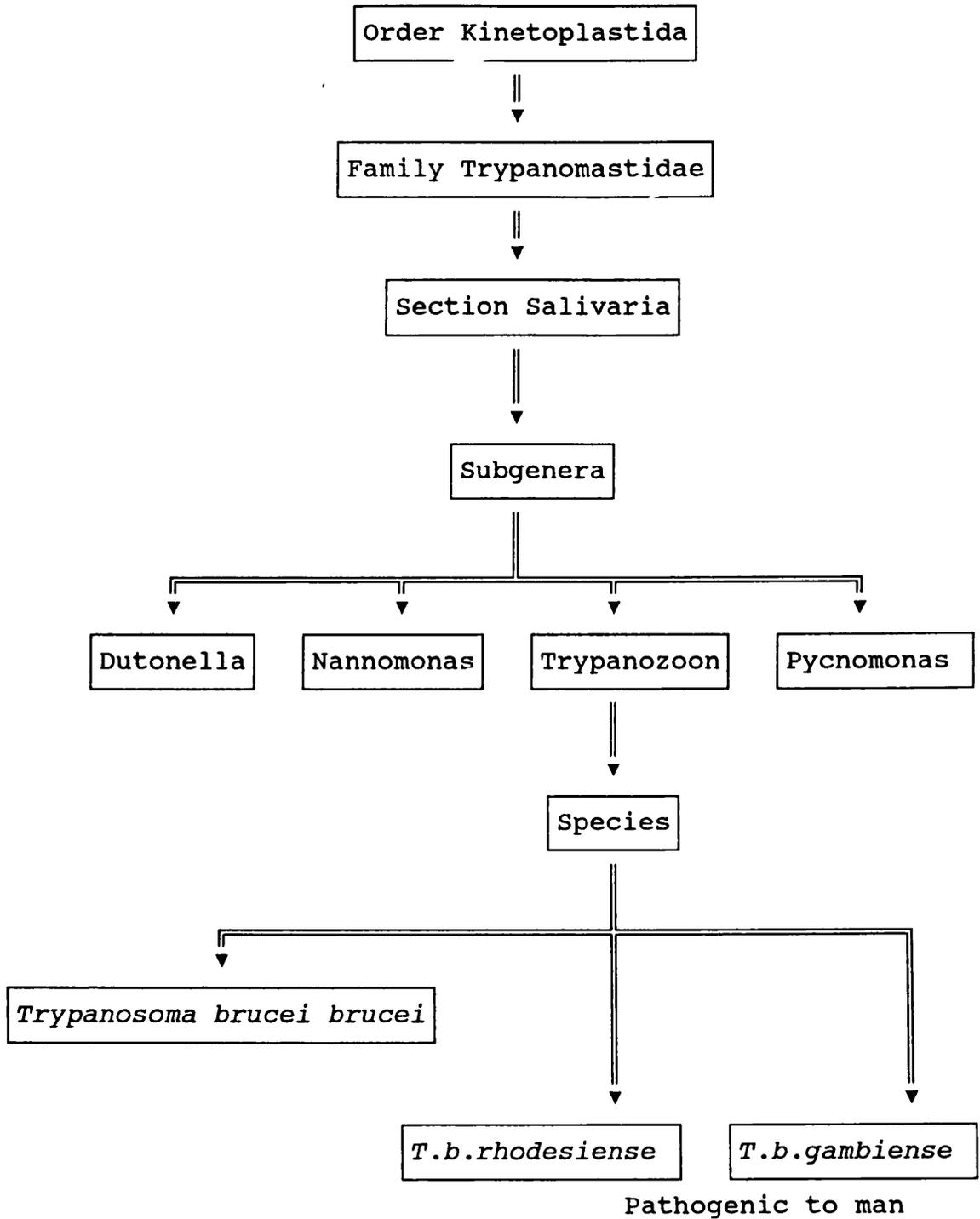
## **1.2 EPIDEMIOLOGY**

### **1.2.1 Aetiology**

The causative organisms of trypanosomiasis in man and animals are various species of *Trypanosoma*, a genus of parasitic protozoa found in the blood and tissues of their hosts. The *Trypanosoma* species that undergo a cycle of development in the tsetse fly are classified as members of the Section *Salivaria*, of the Family *Trypanomastidae*, Order *Kinetoplastida* (Table 1.1). Four subgenera of the salivarian trypanosomes are recognised: *Dutonella*, *Nannomonas*, *Trypanozoon*, and *Pycnomonas*. Human African trypanosomiasis is caused by *Trypanosoma brucei gambiense* and *T.b. rhodesiense* both of which belong to the subgenus, *Trypanozoon*. A third species in this subgenus is *T.b. brucei* which is non-infective in man but causes an acute disease in dogs and

Table 1.1

The classification of the trypanosomes pathogenic to man



horses, and a chronic disease in cattle and pigs, and in experimental animals such as mice (Ormerod, 1970; Hoare, 1972; Molyneux and Ashford, 1983; Jennings, Whitelaw and Urquhart, 1977). The three species of *Trypanozoon* are morphologically indistinguishable (Ormerod, 1970; Hoare, 1972; Molyneux and Ashford, 1983).

### 1.2.2 Transmission

*Trypanosoma brucei gambiense* and *T.b. rhodesiense*, are cyclically transmitted by dipterid flies of the *Glossina* species also called tsetse flies, many of which are capable of transmitting the human infective trypanosomes (Glasgow, 1970).

The *gambiense* form of the disease is mainly found in West and Central Africa. This disease has been reported to be essentially an anthroponosis, transmitted from man to man (reviewed by Jordan, 1986). However, this has been contradicted by the finding that domestic pigs and certain species of wildlife, can harbour *T.b. gambiense* for over 70 days, in a tsetse-infective form (Van Hoof, 1947; Watson, 1962). This suggests that this form of the disease could be an anthrozoosis.

The *rhodesiense* type, found mainly in East and South Africa, is an anthrozoosis frequently transmitted from wild or domestic animals to man. Amongst the trypanosome-susceptible wild animals, the bushbuck, *Tragelaphus scriptus* (Heisch, McMahon and Manson-Bahr, 1958) and the hartebeest, *Aelaphus busephalus* (Geigy, Mwambu and Kaufmann, 1971; Geigy, Mwambu and Onyango, 1972) have been reported to serve as reservoir hosts for *T.b. rhodesiense*.

The association of the trypanosomes with a particular species of tsetse fly is determined by epidemiological characteristics of the disease they cause. Thus, *Trypanosoma brucei gambiense* is transmitted by riverine flies of the *palpalis* group, represented by *Glossina palpalis* and *G.tachinoides* in West

Africa, and by *G. fuscipes* in East Africa. The main vectors of *T.b. rhodesiense* belong to the *morsitans* group represented by *G. morsitans*, *G. swynnertoni* and *G. pallidipes*; these flies inhabit the savanna-like woodlands of East and South Africa, which are abundant in game animals (Hoare, 1970).

However, the suitability of a given species of *Glossina* as a vector depends upon the degree of contact between man and the flies that exist locally. For instance, in North-Eastern Uganda, the vector of *T.b. rhodesiense* is a member of the *palpalis* group (*G. fuscipes*) which usually transmits the *gambiense* form of the disease; at the same time, *T.b. gambiense* has been reported to be transmissible cyclically through *G. morsitans* (Lester, 1933; Corson, 1936), a major vector of the *rhodesiense* form of the disease.

### 1.2.3 Distribution

The tsetse fly infests some 11 million km<sup>2</sup> of Africa (reviewed by Jordan, 1986). The Northern limit is about 14°N and 4°N corresponding closely to the Southern edges of the Sahara and Somalia deserts, respectively. In the South-West it varies from 10°S and 20°S corresponding to the Northern edges of Kalahari and Namibian deserts, whereas in the South-East, it is generally at about 20°S but extends as far as 29°S along the East African littoral (Jordan, 1986).

*Glossina palpalis* and *G. fuscipes*, the chief vectors of the *gambiense* form of sleeping sickness, occur throughout the lowland rain forests and extend well into the drier savanna zones. In the drier parts of their ranges, these *Glossina* species are often associated with surface water and with riparian and lacustrine vegetation (Challier, Gouteux and Coosemans, 1983). The other main vector of *gambiense* sleeping sickness, *G. tachnoides*, occurs along rivers and streams in the savannas of West Africa, although isolated pockets exist in similar vegetation as far East as Ethiopia (Challier *et al*, 1983).

The main vectors of *rhodesiense* sleeping sickness belong to the morsitans group and include *G.morsitans*, *G.swynnertoni*, and *G.pallidipes*. *Glossina morsitans* occurs from Mozambique and Zimbabwe in the South to Tanzania in the North (Hoare, 1970). In these areas, this species infests the extensive 'miombo' woodlands of East Africa and the mopane woodlands in the Zambia Valley. *Glossina swynnertoni* is restricted to *Acacia-Commiphora* vegetation in North Tanzania, extending into Kenya. The third species, *G.pallidipes*, occupies a wide range of thicket and forest edge vegetation in East Africa extending from Ethiopia in the North to Mozambique in the South (reviewed by Jordan, 1986).

These limits of distribution are determined by climate, often through its effect on vegetation. The 500mm isohyet is the limit near the deserts but flies are found in drier areas in the vegetation lining the watercourses (Nash, 1937). Where rainfall is high, the presence of tsetse flies is limited by seasonal low temperatures. The adult flies are inactive at or below 16°C and the puparia do not complete maturation to adult stages below this temperature (Bursell, 1960). On the other hand, puparia of tsetse flies, which develop in the soil, succumb to temperatures above 40-41°C (Bursell, 1960).

Within these general limits, infestations of *Glossina* are not continuous. Extensive areas devoid of trees, naturally or man made, and high grounds are generally tsetse free (Bursell, 1960). For instance, near the equator, the tsetse do not occur above 1,800m above sea level due to the low temperatures of these regions. This altitude decreases with the distance from the equator and is about 1,300m in Zimbabwe. The tree cover would provide shade, reducing the soil temperatures and increasing the survival chances for the puparia (Bursell, 1960).

## **1.3 LIFE CYCLE**

### **1.3.1 In man**

The biting tsetse fly can inoculate metacyclic trypanosomes, during a bloodmeal, into the dermal connective tissue, where they start dividing (Fairbairn and Godfrey, 1957; 1958). A local inflammatory reaction, called a chancre, develops at the site of this initial division. From this site, the trypanosomes enter the draining lymphatics and then the bloodstream. Once in the bloodstream, trypanosomes can traverse the walls of blood and lymph capillaries into the connective tissue and, at a later stage, into the brain and cerebrospinal fluid (CSF) (Fairbairn and Godfrey, 1957; 1958). In all these sites, trypanosomes multiply by binary fission as long slender trypomastigotes, with a mean doubling time of 6 hours (Seed, 1978).

The parasitaemia fluctuates owing to evasion of the host's immune response by antigenic variation. When the host mounts an IgM response to the homotype variable antigenic type (VAT), the parasitaemia goes into remission as trypanosomes of that homotype are killed off (Seed, 1977). Non-dividing, stumpy trypomastigotes replace the slender forms as parasitaemia declines. The stumpy forms can only continue their life cycle in the vector and are killed by the mammalian host's immune mechanisms. Heterotype VATs continue to multiply during the remission of parasitaemia. One of these heterotype VATs overgrows the others to give rise to a recrudescence parasitaemia, in which it becomes the homotype (Van Meirvenne, Janssens and Magnus, 1975). The process continues and gives rise to a chronic infection that is characteristic of African trypanosomiasis.

A particular population of trypanosomes contains a major homotype VAT and several minor heterotype VATs. Each VAT is characterised by a variant-specific glycoprotein (VSG) coat, found only in the mammalian stages and not in the vector stages (Vickerman, 1969; Cross, 1975). The coat protects

the trypanosome from the hosts specific and non-specific defences. Coatless forms activate the complement alternative pathway (Mosser and Robert, 1982; Ferrante and Allison, 1983a) and are readily engulfed and destroyed by macrophages (Mosser and Robert, 1982). In contrast, macrophages do not take up coated trypanosomes except in the presence of VAT-specific antibody (Mosser and Robert, 1982). Many mammals contain natural agglutinins (which also opsonize the parasites for macrophage uptake) to coatless trypanosomes in their serum (Ferrante and Allison, 1983b). Antigenic variation involves the replacement of one VSG coat with another brought about by transcriptional activation of a new VSG gene (Borst and Cross, 1982; Steinert and Pays, 1985). Non-dividing forms, such as the stumpy forms, do not undergo antigenic variation.

Heterotypes arise spontaneously in cloned populations of trypanosomes. Antigenic variation has been reported to take place *in vitro* (Doyle, Hirumi, Hirumi, Lupton and Cross, 1980; Gardiner, Tatthi, Gathuo, Nelson and Moloo, 1986), although the pace of variation is slower than in the vertebrate host. Thus, the host antibody is not the inductive signal for antigenic variation, but can serve to clear specific populations from the blood which are then replaced by the growth of newly arising variants.

### **1.3.2 In the tsetse fly**

The tsetse fly ingests blood into the crop and then the lumen of the midgut where the stumpy trypanosomes transform into the procyclic stage. The slender forms die or change into stumpy forms in the anterior midgut (Vickerman, 1985). Morphological changes of the trypanosomes in the tsetse-anterior midgut include elongation of the post-kinetoplast portion of the body as the simple mitochondrion enlarges and becomes branched. Concomitantly, glycosomes change from spherical to bacilliform structures (Vickerman, 1985). The variable antigenic coat is progressively lost and endocytosis ceases. These

changes occur over a period of 48-72 hours and are accompanied by active division of the flagellated trypanosomes (Steiger, 1973). As the trypanosomes change, there is a switch of energy source from utilisation of glucose, which is scarce in the vector, to proline the source of energy for tsetse flight (Hatson, 1975), and a change from aerobic to anaerobic respiration, as the oxygen tension in the bloodmeal within the tsetse midgut decreases (Bowman and Flynn, 1976).

Four days after ingestion, the procyclics penetrate the peritrophic membrane into the ectoperitrophic space, where they elongate, move forwards to the proventriculus and cease to divide (Hecker, 1980; Vickerman, 1985). They then re-invade the endotrophic space and migrate via the oesophagus to the salivary glands, where they develop into epimastigotes. The epimastigotes attach, with the flagellum, to the microvilli of the epithelial cells lining the salivary glandular lumen. Here, the epimastigotes transform into uncoated trypomastigotes which, like their predecessors, attach to the glandular microvilli and divide. Trypomastigotes then transform into the final stage, the mature metacyclics; these have a variable antigenic coat, lie free in the lumen of the gland, do not divide in the vector and are infective to the mammalian host (Vickerman, 1985).

The mature metacyclic population of a given serodeme of *Trypanozoon* is heterogeneous with respect to VAT (Gray and Luckins, 1976; Steinert and Pays, 1985). A serodeme is a stable, immunologically distinct strain of trypanosomes (i.e has a distinct VAT repertoire), that does not exhibit cross immunity with metacyclic VATs of other serodemes (Van Meirvenne, Magnus and Vervoort, 1977).

## 1.4 THE DISEASE IN MAN

### 1.4.1 Clinical findings

The clinical features of the disease largely depend on the species of the infecting trypanosome. *Trypanosoma brucei rhodesiense* causes an acute to sub-acute illness, with signs of cardiac involvement being most prominent (Manson-Bahr and Charters, 1963; Manuelidis, Robertson, Amberson, Pola and Haymaker, 1965; Francis, 1972; Jones, Lowenthal and Buyst, 1975; Harries and Wirima, 1988). The pre-patent period before the appearance of clinical signs may be as short as 2 weeks (Harries and Wirima, 1988) and the total duration of the disease, from infection to death, may be only 6 weeks (Manuelidis *et al*, 1965). In contrast, *Trypanosoma brucei gambiense* causes a subacute to chronic infection, and although signs of heart damage are encountered (Bertrand, Lobiere, Barabe and Ette, 1971; Adams, Haller, Boa, Doua, Dago and Konian, 1986), the disease is mainly associated with central nervous system involvement (Molyneux, de Raadt and Seed, 1984; Boa, Traore, Doua, Kouassi-Traore, Kouassi and Giordano, 1988).

#### 1.4.1.1 Early stage

Within 5 to 15 days following an infective bite from a tsetse fly, a round inflamed area, of several centimetres in diameter, forms at the site of the bite. This area of inflammation is called a chancre and is characterised by, a red spot surrounded by a waxy zone which appears as a mass of hot and painful minute vesicles (Molyneux *et al*, 1984). It then subsides over a period of 2 weeks to leave an area of scaly desquamation. The chancre is more commonly seen in *T.b. rhodesiense* than in *T.b. gambiense* infections (Molyneux *et al*, 1984).

In *gambiense* sleeping sickness, there are intermittent bouts of fever interrupted, for 2 to 3 days, by periods of lassitude that may last for long durations (years), during which time the patient is unaware of illness. These bouts of fever correspond to the peaks of parasitaemia and trypanolytic crisis

(Onyango, Van Hove and de Raadt, 1966; Apted, 1970). Other initial clinical signs, include, tachycardia, progressive headache, malaise, weakness, an intermittent morbilliform skin rash, and occasional hyperaesthesia (Duggan and Hutchingson, 1966).

In contrast, the *rhodesiense* form may not show these periods of lassitude. Instead, within 10 or so days after the infective bite, there is fever accompanied by headache, weakness, tiredness, occasional rigours and vomiting; generalised oedema with subcutaneous tipping of legs and back. The oedema of the face gives patients a puffy moonface appearance (Manson-Bahr and Charters, 1963; Apted, 1970). Cases of severe diarrhoea have also been reported (Basson, Page and Myburgh, 1977).

The involvement of the CNS occurs faster in the *rhodesiense* form than in the *gambiense* form with CSF showing pathological signs (increased white cell counts and protein) in only 4 or so weeks after infection (Apted, 1970).

Severe congestive heart failure can occur in *gambiense* infections but is more common in *rhodesiense* infections, giving rise to signs such as intermittent tachycardia (Manuelidis *et al*, 1965), dyspnoea even at rest, coughing and occasional haemoptysis (Manson-Bahr and Charters, 1963; Kote and de Raadt, 1969; Francis, 1972), marked venous congestion and hepatosplenomegaly (Manson-Bahr and Charters, 1963). Transient local oedema of the face, and other parts of the body may be seen (Apted, 1970; Wellde, Chumo, Reardon, Mwangi, Asenti, Mbwambi, Abinya, Wanyama and Smith, 1989). Radiography demonstrates cardiomegaly, widening of the superior vena cava and enlargement of hilar vessels, hydrothorax and ascites (Manson-Bahr and Charters, 1963; Francis, 1972; Mbala, Blackett, Mbonifor, Leke and Etoundi, 1988). Abnormal electrocardiographic (ECG) tracings mainly with flattening or inversion of T-waves have been reported in *T.b. rhodesiense*-infections (Manson-Bahr and Charters, 1963; Manuelidis *et al*, 1965; Jones *et al*, 1975)

and in *T.b.gambiense* (Bertrand, Sentilhes, Ducasse, Vacher and Boudin, 1965; Francis, 1972; Mbala *et al*, 1988). In a few patients, there is evidence of conduction defects and of cardiac ischaemia (Bertrand *et al*, 1965; Mbala *et al*, 1988).

Patients develop severe anaemia and lose weight rapidly (Koten and de Raadt, 1969). The anaemia is mostly normocytic and normochromic, sometimes associated with red cell abnormalities, including anisocytosis, polychromasia and hypochromasia (Manson-Bahr and Charters, 1963). The anaemia also involves an increase in the erythrocyte sedimentation rate, consistent with coating of RBCs with antibodies or immune complexes (Molyneux *et al*, 1984). The pathogenesis of anaemia is thought to be multifactorial.

Thrombocytopenia and decreased serum fibrinogen levels are observed in both *T.b.rhodesiense* and *T.b. gambiense* infections accompanied by increased serum and urine levels of fibrinogen degradation products, and decreased prothrombin activity (Barret-Connor, Ugoretz and Braude, 1973; Davis, Robbins, Weller and Braude, 1974; Robins-Browne, Schneider and Metz, 1975; Basson *et al*, 1977; Molyneux *et al*, 1984).

Leukocytes increase reaching maximum levels a few days after peak parasitaemia, mainly due to an increase in mononuclear leukocytes (Ormerod, 1970). Instances of leukocytopenia with a relative lymphocytosis have been observed in *T.b.gambiense* infections (Basson *et al*, 1977). A dominant event is the proliferation of B lymphoid series, either due to deficient T cell control over B cells, or due to the presence of a mitogenic factor for the B cells (Greenwood and Whittle, 1980).

Elevated IgM levels have been found consistently in serum and CSF of human sleeping sickness patients (Mattern, Masseyeff, Michel and Peretti, 1961; Greenwood and Whittle, 1980). IgM levels are raised in laboratory animals infected with the *Brucei* group of trypanosomes (Seed, Cornille, Risby and Gam, 1969; Hudson, Byner, Freeman and Terry, 1976) and in experimental

and natural infections of cattle with these trypanosomes, where the increase over normal may be up to 9-fold (Luckins, 1976). Temporary drops in IgM levels occur and are associated with parasitaemia remission; these might be attributable to parasites mopping up specific IgM (Aoyagi and Tizard, 1976). Careful absorption with a wide range of trypanosome VATs can remove most of this IgM in man (Herbet, Paratti, Van Meirvenne and Lennox, 1980) and in cattle (Musoke, Nantulya, Barbet, Kironde and McGuire, 1981), indicating that IgM production is a specific response to the trypanosomes. In HAT, IgG and IgA levels are in the normal range but IgE levels are raised Herbert *et al*, 1980). The presence of auto-antibodies has been reported (Blackett and Ngu, 1976; Mbala *et al*, 1988).

There is also an increase in serum levels of immunoglobulins (Blackett and Ngu, 1976; Basson *et al*, 1977; Lambert, Berney and Kazyumba, 1981; Mbala *et al*, 1988) accompanied by a decrease in the total hemolytic complement, serum C3 and C4, with indications of high C3 catabolism (Blackett and Ngu, 1976; Mbala *et al*, 1988). Severe hypoproteinaemia and hypoalbuminaemia have also been described (Jenkins and Robertson, 1959; Korten and de Raadt, 1969).

Hepatomegaly and hepatic dysfunction reflected by increased serum bilirubin, alkaline phosphatase (AP), alanine transferase (ALT) and slight to severe jaundice, have been reported (Robertson and Jenkins, 1959; Apted, 1970; Basson *et al*, 1977). Partial renal dysfunction characterised by low urine volume, high urinary sodium content, mild proteinuria, haematuria and  $\alpha$ -ketoaciduria have also been observed (Hawking and Greenfield, 1941; Poltera, Owor and Cox, 1977; Basson *et al*, 1977). Blood urea nitrogen and creatinine vary from normal to increased concentrations (Barret-Connor *et al*, 1973). Enzyme alterations related to muscular involvement have been reported (Barret-Connor *et al*, 1973; Basson *et al*, 1977)

Endocrine dysfunction manifests as amenorrhoea, abortion caused by uterine hypoplasia, premature births and perinatal deaths in women; and as impotence and, in later stages of the disease, as gynaecomastia in men (Poltera, 1985). Orchitis has been observed in *rhodesiense* infections of man (Losos and Ikede, 1972; Welde *et al*, 1989) and in experimental *T.b.brucei* infections of dog (Morrison, Murray, Sayer and Preston, 1981a).

The eyes may show iridocyclitis with variable degrees of keratitis, circumcorneal infection, conjunctivitis and photophobia (Apted, 1970).

#### **1.4.1.2 Late-stage**

The main clinical signs that occur during the late-stage are related to the CNS involvement and include, fasciculation of muscles of the limbs, face, lips and tongue, oscillatory movements of the arms, head, neck, or trunk especially in children, and an increase in tonicidity or muscular rigidity. There is usually a considerable element of cerebellar ataxia. Focal lesions may cause transient or more commonly permanent paralysis of certain groups of muscles (Gallais, Collomb, Miletto, Dutertre and Berardbadier, 1956). Epileptiform convulsions may occur depending on the area of the CNS affected.

The commonest and most characteristic sign is daytime somnolence which starts as an occasional nod and progresses to continuous sleep. However, a small portion of patients suffer insomnia (Welde *et al*, 1989). At this time, speech is slow and indistinct, there is hypothermia, pruritis intensifies and severe emaciation occurs. Sleep progresses into coma, sphincter control is lost and death follows often from secondary intercurrent diseases such as pneumonia (Welde *et al*, 1989). Personality alterations are manifested as mental dullness that progresses to apathy, lethargy and indifference to surroundings. Emotional disturbances are also common and include, laughter, crying or outbursts of rage, episodes of manic behaviour sometimes with hallucinations and delirium (Tooth, 1950; Lambo, 1966). These changes occur mainly in *gambiense*

infections.

These late-stage features of CNS involvement are rarely seen in *rhodesiense* sleeping sickness because patients die before such signs develop. However, they can occur following unsuccessful therapy with early-stage drugs such as pentamidine (Apted, 1970).

It is difficult to make a sharp demarcation between the clinical signs of early- and late-stage sleeping sickness and there is little documented clinical information on the early stage disease since most patients are diagnosed in the late stages. A recent study in Uganda highlighted body weakness, somnolence, inability to stand and walk unaided, disturbance of speech and incontinence as the most constant presenting symptoms of late-stage *rhodesiense* sleeping sickness; while hand and tongue tremors, ataxic gait, loss of Babinsky reflex and positive cheiro-oral reflex are the most commonly occurring symptoms in late-stage *rhodesiense* sleeping sickness (Mbulamberi, 1989). By contrast, headache, fever, joint pains, insomnia, inability to work normally and loss of appetite were significantly more common complaints among early-stage sleeping sickness patients (Mbulamberi, 1989).

#### **1.4.2 Pathology**

The pathology of human African trypanosomiasis exhibits a wide range of variation from the fleeting parasitaemia caused by a strain derived from animals (Van Hoof, 1947), through the heavy parasitaemia of the virulent *T.b. rhodesiense* disease, the insidious *T.b. gambiense* infection which can exterminate a whole population, to the unusual balanced situation noted in Nigeria (Duggan, 1962) and Sierra Leone (Harding and Hutchingson, 1948) where high rates of infection are associated with mild symptoms and a death rate similar to that observed in trypanosome free areas.

#### 1.4.2.1 Systemic circulation and tissues other than the central nervous system

The chancre is a hard, painful, red nodule of several centimeters in diameter, which is similar to the cellulitis seen around a staphylococcal boil but lacks pus, and represents the first major response of the host to the trypanosome. The histology of a chancre reveals edema, trypanosomes and perivascular infiltration by lymphocytes (Fairbairn and Godfrey, 1957; 1958). A similar and more detailed description of the gross and histopathological changes of the chancre has been reported in cattle experimentally infected with *T.b. brucei*, *T.vivax* and *T. congolense* (Murray and Morrison, 1980; Akol and Murray, 1982), and in goats infected with *T.brucei* and *T.congolense* (Emery, Akol, Murray, Morrison and Mooloo, 1980). In their description, trypanosomes are readily identified in the dermis accompanied by intense inflammatory reaction, followed by accumulation of large lymphoid cells and plasmablasts. These changes result in the disruption of the dermal collagen, causing fragmentation. As the lesion declines in size, increased numbers of mature plasma cells, macrophages, eosinophils and mast cells are found (Murray and Morrison, 1980; Emery *et al*, 1980; Akol and Murray, 1982).

Following the enlargement of the lymph nodes draining the chancre, generalised lymph node enlargement occurs. Histology of lymph nodes taken from patients dying during the acute phase of the disease, show that the enlargement is partly due to a proliferative lymphoid cell response. The lymphoid germinal centers undergo changes in composition from clusters of small lymphocytes to cells of the lymphocyte-plasma cell series, accompanied by an infiltration by macrophages (Ormerod, 1970). The presence of trypanosomes may be microscopically evident since aspirations of swollen lymph nodes is one of the most reliable methods of isolating trypanosomes from suspected cases. Similar changes have been described in experimental infections

of cattle with *T.b. brucei*, *T.congolense* and *T. vivax* (Murray and Morrison, 1980; Akol and Murray, 1982), of goats infected with *T.b. brucei* and *T. congolense* (Emery *et al*, 1980), and of dogs infected with *T.b. brucei* (Morrison, Murray, Sayer and Preston, 1981c). In addition to these changes, the lymph nodes of dogs experimentally infected with *T.b. brucei*, show an expansion of both the cortical and medullary regions, petechial, and sometimes ecchymotic haemorrhage (Morrison *et al*, 1981c); numerous IgM-containing cells are found mainly around the follicles, but a few of these may be found within the follicles; the lymph node sinuses are extremely oedematous and distended with, in chronological order of appearance, small lymphocytes, large lymphocytes, macrophages, trypanosomes and fibrin deposits (Morrison *et al*, 1981c).

As the disease progresses in man, fibrosis of the lymph vessels and nodes occur making lymph nodes small and sometimes hard. During this transition they may contain morular or Mott cells, also called Russell-body containing cells (Ormerod, 1970), which are modified plasma cells that produce, but are unable to secrete immunoglobulins. The immunoglobulin-filled vacuoles distend the cytoplasm giving it a characteristic bunch-of-grapes appearance (Low and Mott, 1904). There are numerous vacuolated macrophages and Mott cells throughout the cortex; the germinal centers show a loose cellular arrangement and a decreased lymphocytic content (Ormerod, 1970; Poltera, 1985). The sinuses are distended with macrophages, and bi- and multinucleate giant cells (Ormerod, 1970). Similar changes have been shown to occur in *T.b. brucei*-infected dogs (Morrison *et al*, 1981c; Ndung'u, 1990) cattle (Morrison, Murray, Whitelaw and Sayer, 1983), mice and rats (Murray, Jennings, Murray and Urquhart, 1974b).

In the early stages of sleeping sickness, there is a gradual fall of both red and white blood cells with differential white cell count showing a relative fall in

granulocytes and a rise in mononuclear cells (Ormerod, 1970). Proliferation of B lymphocytes is a dominant pathological event that first takes place in the parasitised lymph nodes and is associated with the appearance of large amounts of IgM and autoantibodies in the serum (Greenwood and Whittle, 1980). It has been suggested that the exhaustion of the B lymphoid system by the sustained trypanosomal stimulus contributes to immunodepression in man (Greenwood and Whittle, 1980). In experimental infections, parasite specific immunosuppression has been found to be transient in early stages and complete in the advanced disease (Dempsey and Mansfield, 1983). Polymorphonuclear cells seldom occur in the reaction of man to trypanosome infection and when they occur, are usually associated with secondary bacterial infections (Ormerod, 1970; Greenwood and Whittle, 1980). However, in experimental infections polymorphonuclear cells are consistently found in the tissue lesions in dogs (Morrison *et al*, 1981b; Ndung'u, 1990) and occasionally in mice and cattle (Morrison *et al*, 1983; Jennings, McNeil, Ndung'u and Murray, 1989).

There is an increase in immunoglobulin production as shown by the rise in erythrocyte sedimentation rates (Gall, Hutchinson and Yates, 1957). The continuous accumulation of IgM in the blood and the insignificant secondary IgG increase, is unusual compared to other infections (Mattern, 1962). IgM is a large molecule that is unable to cross membranes efficiently, which may explain the presence and persistence of trypanosomes in areas with blood-tissue barriers such as the testes (Clapier, 1921), and the brain (Jennings and Gray, 1983). As the disease progresses, the level of immunoglobulins in blood remains high but there may be immunological failure characterised by the absence of complement, the occurrence of pseudo-positive Wassermann reactions and the presence of heterophile antibodies (Henderson-Begg, 1946; Greenwood and Whittle, 1980; Poltera, 1985).

The spleen increases in size in proportion to anaemia, becoming firm and cellular, with fibrosis occurring only at the very late stages of the disease. The

sinusoids contain activated macrophages in the early stages (Gall, Hutchingson and Yates, 1957; Greenwood and Whittle, 1980). There is an increase in the number of malphigian corpuscles, accompanied by a change in their cellular composition, from clusters of small lymphocytes to cells of the lymphocyte-plasma cell series (Ormerod, 1970). The changes that occur in the spleen during trypanosome infections have been closely studied in experimental animals. Thus, experimental infections with *T.b. brucei* in dogs (Morrison *et al*, 1981c), and rats (Murray, Jennings, Murray and Urquhart, 1974a; 1974b) show that the periarteriolar regions of the spleen contain numerous lymphoblasts, some plasma cells and relatively few small lymphocytes: large active germinal centers containing heavy granular deposits of IgG and IgM are found in majority of follicles with numerous macrophages and a prominent layer of small lymphocytes. Disruption of the lymphoid cellular arrangement occurs due to accumulation of red blood cells, deposition of fibrin, and infiltration by polymorphonuclear leukocytes, often accompanied by localized necrosis of the lymphoid cells. There is marked increase in the number of highly mitotic plasma cells and large lymphocytes in the red pulp, the cords of Billroth and a less marked increase in the sinuses. As the infection progresses there is an increasing number of macrophages in the sinuses some of which contain phagocytic granules. Megakaryocytes are reported to occur occasionally accompanied by a small number of erythropoietic cells, a sign of extramedullary erythropoiesis (Morrison *et al*, 1981c).

In the late stages of sleeping sickness, the periarteriolar regions of the spleen become less active and show a higher content of small lymphocytes. The follicular areas become disorganized with few discrete active germinal centers. Most of the follicular areas are depleted of lymphocytes and contain macrophages in what looks like the remains of previously active germinal centers. In the red pulp the plasma cell content is reduced and occasional Mott

cells can be found. The sinuses and cords contain numerous active macrophages, many of which are involved in erythrophagocytosis. Focal fibrin deposits and thrombi are common in the sinusoids and, occasionally, in the branches of the splenic vein (Ormerod, 1970; Poltera, 1985). In dogs, the late-stage disease is characterised by a widespread presence of megakaryocytes and foci of erythropoietic cells in the red pulp, the sinuses and the cords of the spleen (Morrison *et al*, 1981c; Ndung'u, 1990).

The heart is considered to be the organ most at risk in the early stages of *T.b. rhodesiense* infection, e.g., the major cause of mortality in the Ugandan *T.b. rhodesiense*-epidemic of the 1940s was concluded to be myocarditis. Severe myocarditis has been reported in both *rhodesiense* (Hawking and Greenfield, 1941) and *gambiense* sleeping sickness (Mott, 1906). Histologically, a pancarditis involving all cardiac layers including valves and conducting system has been reported (Lavier and Leroux, 1939; Poltera, Cox and Owor, 1976). The pancarditis consists of a focal or diffuse mononuclear cell infiltration of the interstitium and the perivascular spaces (Poltera, Cox and Owor, 1976), myocytolysis, and focal endomyocardial fibrosis (Hawking and Greenfield, 1941; Cohen, 1973; Poltera *et al*, 1977). This is the basis for the clinically reported cardiac insufficiency and electrocardiographic changes (Lavier and Leroux, 1939; Poltera *et al*, 1976). In human infections, the involvement of the autonomic nervous system of the heart has also been observed (Poltera *et al*, 1977).

In experimental infections in various experimental animals, myocarditis has been reported to be composed of focal haemorrhage, edema and separation of muscle fibres, extravascular trypanosomes in association with local inflammatory infiltrates composed of, mainly, lymphocytes, plasma cells and occasional foci of macrophages. This is the typical lesion reported in *T.b. brucei*-infected dogs (Morrison *et al*, 1981b; 1983; Ndung'u 1990), rats (Murray *et al*, 1974a; 1974b), mice (Murray *et al*, 1974a; 1974b; Poltera, 1980), and in

*T.b. rhodesiense*-infected vervet monkeys (Peruzzi, 1928; Poltera and Sayer, 1983). In addition to these changes, polymorphonuclear leukocytes are a consistent finding in the lesions of the dog (Morrison *et al*, 1981a; Morrison *et al*, 1983; Ndung'u 1990). In the dog, the atria are affected to a greater extent than the ventricles and the infiltrations are more marked in the subepicardial and perivascular locations (Ndung'u, 1990).

Cardiac aneurysms from trypanosome endemic areas of Africa have been described (Poltera *et al*, 1976). Cardiomyopathies and endomyocardial fibrosis have been found to correlate with autoantibody titres in *T.b. gambiense* and *T.b. rhodesiense* infections in man (Blakett and Ngu, 1976; Poltera and Cox, 1977, respectively). In experimental *T.b. rhodesiense*-infections in vervet monkeys, apical aneurysms have also been reported, which are thought to be a consequence of the partial obstruction of the cardiac lymphatic drainage (Poltera and Sayer, 1983). In terminal stages of experimental *T.b. brucei* infection in dogs (Morrison *et al*, 1981b) and rats (Murray, Murray, Fisher and Urquhart, 1974), large numbers of trypanosomes are found scattered diffusely throughout the myocardium and numerous foci of myocardial cell degeneration and necrosis occur. These changes are associated with marked infiltration with lymphocytes, plasma cells and many macrophages and, in the case of dogs, polymorphonuclear leukocytes.

Hepatic dysfunction has been shown to occur during the course of disease (Jenkins and Robertson, 1959) and jaundice has been reported (Gelfand and Friedlander, 1963). Cloudy swelling and centrilobular necrosis are common findings in the liver. Whether these are due to trypanosome infection or are a result of secondary infections and terminal state is not clear (Ormerod, 1970). In *T.b. brucei*-infected dogs (Morrison *et al*, 1981b), the hepatic sinusoids are markedly distended and contain large numbers of plasma cells, lymphocytes, variable numbers of polymorphonuclear leukocytes and trypanosomes. Kupffer

cells increase in number and occasionally contain phagocytosed erythrocytes. Foci of fatty degeneration occur in close proximity to the hepatic veins. Swelling and disorganisation of hepatocytes with occasional cell death and mitosis may occur.

In summary, the most consistent findings are lymphoid hyperplasia, infiltration of tissues (including, the heart and the brain), by trypanosomes and cells of lymphocyte/plasma series and macrophages, hypergammaglobulinaemia, progressive anaemia, leucopenia and immunosuppression, and great wasting of the limb, abdominal and thoracic muscles.

#### **1.4.2.2 Central nervous system**

It is not clear when the meningeal reaction starts. However, the appearance of cells, immunoglobulins and protein in the CSF from cisternal puncture (Neujean, 1950), as well as the involvement of the choroid plexus suggest that cerebral involvement occurs early and progressively (Poltera, 1985). Le Port (1935) and Calwell (1937) produced the first evidence that mental disturbances and headaches were accompanied by swelling of the choroid plexus, which blocked the lateral apertures of the fourth ventricle and of the interventricular foramen; and led to congestion of cerebral blood vessels. Meningocortical infiltration by lymphocytes and plasma cells correlates with early stages of CNS involvement, whereas, cerebral white matter and basal ganglia lesions are involved in later stages (Manuelidis *et al*, 1965; Poltera *et al*, 1976). Perivascular cuffing does not appear to occur until at least 3 months after infection, and is accompanied by the presence of small round cells (probably infiltrating lymphocytes or proliferating microglia) in the brain parenchyma (Mott, 1906; Calwell, 1937; Van Bogaert, 1958; Manuelidis *et al*, 1965; Ormerod, 1970; Poltera *et al*, 1976).

Several gross anatomical descriptions of the CNS lesions in sleeping sickness were published in the nineteenth century. The most complete work was

produced by Clark (1840) who described the inflammation of the brain associated with swelling and adhesion of the dura to the arachnoid mater. Meningoencephalitis was first observed by Mott in 1899. He (Mott, 1906) and much later, Van Bogaert (1958) published detailed descriptions of the CNS lesions in *gambinese* sleeping sickness, While Calwell (1937) published a detailed account of *rhodesiense* sleeping sickness.

The inflammation affects mainly the pia-arachnoid mater (Ormerod, 1970) and the choroid plexus (Calwell, 1937). The typical histological lesion is a vasculitis with perivascular infiltration of the leptomeninges and Virchow-Robin spaces with mononuclear inflammatory cells, including, lymphocytes, plasma cells and macrophages with occasional Russell body-containing plasma cells. These cells extend into the adjacent parenchyma in severe cases of the disease (Mott, 1906; Calwell, 1937; Van Bogaert, 1958; Manuelidis *et al*, 1965; Poltera *et al*, 1976; Poltera, 1985; Adams *et al*, 1986). The pathology slowly spreads to the remote parts of the subarachnoid space. Thus, only in advanced cases are Russell body-containing plasma cells obtained at lumbar puncture (Mott, 1906). The most characteristic lesion resulting from this spread is the formation of perivascular cuffs whereby the Virchow Robin spaces are infiltrated by the mononuclear cells. This may consist of a single layer of cells or in advanced cases, a 20-30 cell-deep sheath. Russell body-containing plasma cells are present in all the inflammatory foci in the CNS, which led to their presence in the perivascular cuffs being erroneously considered to be pathognomonic of sleeping sickness (Mott 1906).

Microglia increase in number, in the brain parenchyma, preceding the formation of perivascular cuffing, and infiltrating the cuffs as they form (Mott, 1906; Calwell, 1937; Van Bogaert, 1958; Manuelidis *et al*, 1965; Ormerod, 1970; Poltera *et al*, 1976). Poltera *et al* (1976) and Manuelidis *et al* (1965) emphasized the prominence of swollen astrocytes and the absence of neuronal

damage. In the very advanced perivascular cuffing, chromatolysis of neurones around the cuffed vessels, occasionally leading to Wallerian-like degeneration of nerve tracts, occurs (Mott, 1906; Calwell, 1937; Van Bogaert, 1958; Manuelidis *et al*, 1965; Ormerod, 1970; Poltera *et al*, 1976). Swollen masses of macroglial cells may be seen where neuronal degeneration occurs.

Topographically, the lesion is most evident in the white matter of the cerebral hemispheres, the basal nuclei, the white matter of the cerebellum and the brain stem (Calwell, 1937; Poltera *et al*, 1977; Adams *et al*, 1986; Haller, Adams, Merouze and Dago, 1986). Occasionally the lesion extends to the deeper layers of the cerebral cortex. In more florid cases, there are collections of lymphocytes, plasma cells and microglia in the brain not directly related to blood vessels. Diffuse microglial hyperplasia in the grey matter and in a few cases large reactive astrocytes in the white matter have also been observed (Adams *et al*, 1986; Haller *et al*, 1986). Russell body-containing plasma cells occur within perivascular spaces and in association with groups of cells of inflammatory type in the white matter. Isolated Russell body-containing plasma cells have been reported to occur in areas that in all other respects appear normal (Adams *et al*, 1986).

The involvement of the choroid plexuses and the cranial nerves has been reported (Calwell, 1937; Poltera *et al*, 1977). However, Adams *et al* (1986) observed that inflammatory processes in the ventricles or in the choroid plexus occurred only occasionally and that encephalitis was more common than meningitis.

In summary, the consistent pathological features of CNS trypanosomiasis are lymphocytic-plasmacytic meningoencephalitis, especially in a perivascular distribution, glial proliferation and sparing of the neuronal elements. In some fatal cases, chromatolysis of the neurones and degeneration of the nervous tracts, and choroid plexus involvement occur. Occasionally a haemorrhagic encephalopathy occurs especially after arsenical therapy.

## 1.5 CONTROL OF HUMAN AFRICAN TRYPANOSOMIASIS

The control of sleeping sickness relies on an integrated strategy of continuous surveillance, involving, diagnosis and treatment of the population at risk, and vector control, where applicable (de Raadt, 1986).

Vector populations can be controlled for a time by the application, from air or ground, of suitably formulated insecticides, a very costly and potentially ecologically harmful venture. Nowadays, more attention is being given to cheaper and more ecologically friendly methods, applicable at community level. These include, modification of the habitat by bush clearing around human habitation and rivers, and the use of odour-baited, insecticide impregnated traps and targets (WHO, 1986). Indeed, the control of tsetse flies by means of simple traps with community participation has been shown to be effective in the control of the sleeping sickness epidemic in Uganda (Lancien, 1991).

A number of new tools, particularly for diagnosis and vector control have been developed (Cattand and de Raadt, 1991; Nantulya, Doua and Molisho, 1992; Komba, Odiit, Mbulamberi, Chimfwembe and Nantulya, 1992). Despite the fact that chemotherapy is the major means of disease control, development of new anti-trypanosomal drugs has been more or less static over the last three decades, due to lack of interest by the pharmaceutical industry to invest into research and development of trypanocides (Gutteridge, 1985). The methods of diagnosis and the drugs available for treatment of HAT are considered below.

### 1.5.1 Diagnosis

The standard method for diagnosis of trypanosomiasis is to demonstrate and identify trypanosomes in body fluids, including, blood, chancre fluid, lymph node aspirates, and CSF. Owing to the tissue invasiveness of the causative agents of HAT, *T.b. rhodesiense* and *T.b. gambiense*, and the intermittent

nature of the infections they cause, it is not always possible to demonstrate parasites in body fluids. Moreover, in the insidious *gambiense* infection, several months may elapse before the clinical manifestations develop; and even when they do, the manifestations are not pathognomonic (Losos and Ikede, 1972). The techniques employed for diagnosis of HAT can be divided into three major groups: parasitological, immunological and molecular.

#### **1.5.1.1 Parasitological diagnosis**

The easiest technique for detection of trypanosomes in peripheral blood is by direct microscopy of blood, either the wet film method to detect motile trypanosomes or, as stained thick and thin smears when parasites are identified on the basis of their morphology by light microscopy (WHO, 1986). Examination of wet blood films is quick and suitable for screening large numbers of samples but is too insensitive as half of the infected patients may not show parasites (Barnet, 1947; Baker, 1970; WHO, 1986). Trypanosomes can sometimes be demonstrated by direct microscopy of aspirates from the enlarged cervical lymph nodes, chancre or cerebrospinal fluids (WHO, 1986). In suspected cases where the presence of parasites cannot be demonstrated in blood, lymph fluid or CSF, examination of stained bone-marrow aspirates has proved to be a sensitive technique (WHO, 1986).

The haematocrit centrifugation technique (HCT) improves the sensitivity of direct microscopic examination, by concentrating the parasites (by centrifugation) in unclotted blood at the buffy coat (Woo, 1969; Murray, Murray and McIntyre, 1977; Kelley and Shillinger, 1983). A modification of this method to increase its sensitivity by including a plastic float, has been described (Levine, Wardlaw and Patton, 1989). The HCT is particularly useful in that the status of anaemia in the test subject can be assessed at the same time. This is a simple and rapid method that is applicable far afield with the advent of battery operated microfuges.

The miniature anion-exchange centrifugation technique (mAECT) which removes the red cells from a blood sample by trapping them in a diethylaminoethyl (DEAE) cellulose column followed by concentration by centrifugation, is widely used to diagnose *T.b.gambiense* infections in man (Lumsden, Kimber and Strange, 1977; Lumsden, Kimber, Evans and Doig, 1979). In another method, fractionation is achieved by centrifugation of blood through silicone layers. Other methods of removing red blood cells by lysis using hypotonic shock or detergent, followed by centrifugation, may gain wider application due to their simplicity (WHO, 1986). Single and double centrifugation, has been shown to substantially increase the possibility of finding trypanosomes in the CSF (Cattand, Miezán and de Raadt, 1988).

The most sensitive method of parasitological diagnosis is sub-inoculation of test blood or CSF samples into susceptible laboratory animals, followed by examination of blood for development of parasitaemia (reviewed by Paris, Murray and McOdimba, 1982). This method has an added advantage in that trypanosome isolates can be collected for further studies. The delay in diagnosis and the cost of maintaining laboratory animals makes it impractical for routine diagnosis. Furthermore, many *T.b. gambiense* isolates do not infect laboratory animals. Isolation of parasites from aparasitaemic patients by an *in vitro* technique has also been described (Aerts, Truc, Penchemier, Claes and Le Ray, 1992). Further evaluation of this technique is currently going on.

#### **1.5.1.2 Immunological diagnosis**

The tests that detect antibodies in the sera and CSFs of trypanosomiasis patients include: capillary tube agglutination test - CAT (Ross, 1971), immunoprecipitin tests (Aiyedun, Amodu, Bidwell, Bone, Buck, Coulm, Frezil, Kent, Mattern, Njogu, Voller and Wery, 1976; Taylor and Smith, 1983), indirect immunofluorescence antibody test, IFAT (Wery, Van Wetter, Wery-

Paskoff, Van Meirvenne and Mesatewa, 1970; Katende, Nantulya and Musoke, 1987), indirect haemagglutination test, IHAT (Bone and Charlier, 1975), enzyme-linked immunosorbent assays, ELISA (Voller, 1977; Luckins, Gray and Rae, 1978, Luckins, Boid, Rae, Mahmoud, El Malik and Gray, 1979) and card agglutination test for trypanosomiasis, CATT (Magnus, Vervoort and Van Meirvenne, 1978). These tests detect antibodies and, therefore, cannot distinguish between active and cured infections. The CATT (Magnus *et al*, 1978) is the most reliable of these methods and utilizes variant surface antigens of selected trypanosome antigenic variants which are stabilised on the whole trypanosome. The other tests utilise antigens of undefined specificity and purity, making them difficult to standardise (WHO, 1986).

Following the observation that the trypanosome procyclics' surface antigens are species or subgenus specific, various antigen detection immunoassays have been developed (Nantulya, Musoke, Rurangirwa, Minja and Saigar, 1985; Parish, Morrison and Pearson, 1985; Richardson, Jenni, Becroft and Pearson, 1986; Liu and Pearson, 1987; Nantulya, Musoke, Rurangirwa, Saigar, and Minja, 1987; Nantulya, 1989). These tests detect specific, trypanosome surface antigens, in sera and CSF of infected patients and should differentiate between current and past infections (WHO, 1986).

#### **1.5.1.3 Molecular diagnosis**

This method is based on the detection of parasite DNA from the blood, CSF, lymph node aspirate, bone marrow or tissues. Within the parasite genome, there are repeated DNA sequences, known as 'satellite' DNAs, which have no known cellular function and typically contain a simple sequence that is repeated thousands and often millions of times. In *T.brucei* and *T.evansi*, these elements constitute 12% and 9% of the genome, respectively (Borst, Face-Fowler, Frasch, Hoeijmakers and Weijers, 1980; Castro, Craig and Castaneda, 1981; Sloof, Bos, Konings, Borst, Gutteridge and Leon, 1983a; Sloof, Menke,

Caspers and Borst, 1983b; Koehavong and Thilly, 1989). The satellite DNA contains the most convenient parasite species-specific differences, which can be used for diagnosis.

DNA hybridization can also be used for diagnosis. A single-stranded DNA fragment, containing specific DNA sequences, such as parasite species-specific sequences, is identified, purified and labelled with a tracer. The labelled fragment is then used to probe whole parasite DNA or the whole organism. The test parasite DNA is split into single strands using denaturing reagents so that when the probe is applied to the specimen, these strands anneal to complementary DNA sequences of the parasite. The bound label can then be revealed by dye development or autoradiography depending on the label used (WHO 1986).

Recently, amplification of the satellite DNA by the polymerase chain reaction was introduced (Moser, Cook, Ochs, Barley, McKane and Donelson, 1989) and has been reported to be very sensitive, detecting upto one tenth of a parasite.

### **1.5.2 Chemotherapy**

Many drugs mainly arsenicals and antimonials, have been used in the treatment of sleeping sickness. Atoxyl, one of the first to show promise, was introduced in 1905 but unfortunately, its use was sometimes followed by optic nerve atrophy (reviewed by Hawking, 1963). Tartar emetic appeared a few years later and was effective but relapses after use were frequent. Tryparsamide became available in 1919 and suramin (Bayer 205), developed some years earlier, was first used in sleeping sickness in 1922. Other drugs in great variety, including the diamidines introduced in the 1930s, have been used, but of the early drugs only suramin and tryparsamide have survived, although tartar emetic may occasionally be of value if other drugs are not available. Melaminyl

(arsenical) compounds introduced in 1939 tended to supersede trypanamide except in areas with special problems where the latter is still of use (Apted 1970). The drugs can be divided into two major groups: those that cannot cross the blood-brain barrier and can therefore treat only the early stages of the disease, including, suramin, pentamidine and Berenil, and those that cross the blood-brain barrier and are required to treat the patients with late-stage central nervous system involvement, such as melarsoprol and eflornithine.

### 1.5.2.1 Early-stage drugs

#### 1.5.2.1.1 Suramin

Suramin is the symmetrical 3 urea sodium salt of 8-(3-benzamido-4-methylbenzamido)naphthalene-1,3,5,-trisulphonic acid. It is administered intravenously (i.v.) at 20mg/kg single dose (adult dose, maximum adult dose is 1g). The dose is repeated five times at 5-7-day intervals. Suramin is trypanocidal and parasites disappear from blood and lymph nodes of patients within a few hours of its administration (Apted, 1970). It is equally effective against both *gambiense* and *rhodesiense* infections. Suramin remains unique amongst trypanocides as it is negatively charged at physiological pH and as such readily binds to serum proteins. In a few patients, suramin fails to reach therapeutic concentrations in blood (Hawking, 1940). Suramin inhibits a multitude of enzymes in cell free systems at millimolar concentrations amongst which L-alpha glycerolphosphate oxidase, an enzyme concerned with oxidation of reduced nicotinamide diphosphate (NADPH) in African trypanosomes, is markedly sensitive (Fairlamb and Bowman, 1980). Although it permeates the blood-brain barrier poorly, some effect on early nervous system infections have been reported (Keevil, 1934).

A febrile reaction to the first or the second injection is almost invariable. In a few patients, injection is followed by vomiting, shock and collapse (Apted, 1970). Other early signs of toxicity, include, pruritis, urticaria, a papular

eruption, conjunctivitis, photophobia, palpebral oedema and stomatitis. Cutaneous hyperaesthesia may be noted and particularly pain in the soles of the feet which may be so severe as to cause difficulties in walking. The commonest toxic effect is some degree of renal damage resulting in mild albuminuria. However, suramin is one of the most widely used and one of the safest trypanocides. It forms complexes with plasma proteins and is excreted very slowly so that a single injection of 1g can maintain prophylactic concentrations in blood for about three months.

#### 1.5.2.1.2 Pentamidine

This aromatic diamidine is present in two forms: pentamidine isethionate and the related methanesulphonate. It is administered at 3-4mg/kg everyday for 7 days. The single maximum dose is 300-400mg. Pentamidine is trypanocidal and is very effective in the early stages of *gambiense* sleeping sickness. It does not cross the blood-brain barrier and therefore has no effect on the CNS infections. Its effects are less certain in the *rhodesiense* form of the disease where relapses and severe deterioration of the patients have been reported in East and West Africa (Apted, 1970; Hutchinson and Watson, 1962).

Histamine is released following intramuscular administration of pentamidine with subsequent hypotension, dizziness and occasional collapse. The drug also causes hypoglycaemia (Williamson, 1970; Gutteridge, 1985). Pentamidine is a valuable prophylactic and a single dose can protect against *gambiense* infection for at least six months, but its prophylactic value in *rhodesiense* form is less certain (Williamson, 1970).

The mechanism of action of aromatic diamidines is still incompletely resolved. They are positively charged and bind to DNA exerting a marked effect on biosynthesis of nucleic acids (Gutteridge, 1969; Newton, 1974). The synthesis of kinetoplast DNA is particularly sensitive to disruption by diamidines

(Brack, Delan, Riou and Feisty, 1972). They also inhibit S-adenosylmethionine (SAM)-decarboxylase (Bitonti, McCann and Sjoerdsma, 1986), an enzyme involved in the formation of decarboxylated SAM (dSAM) from SAM, which in turn makes spermidine from putrescine during the synthesis of glutathione, in mammals, and of trypanothione in the trypanosome (Henderson and Fairlamb, 1987).

#### **1.5.2.1.3 Berenil**

Berenil (diminazene aceturate), like pentamidine, is an aromatic diamidine that has been shown to have marked activity in a variety of trypanosome infections (but not *Trypanosoma cruzi*) and the veterinary parasitosis, babesiosis (Fussganger and Bauer, 1958; Whiteside, 1962a;1962b; Fairclough, 1963a; 1963b; Van Hove and Grainge, 1966, MacLennan, 1968; Fink and Dann, 1974). Although Berenil is registered as a veterinary drug, its successful use in the early stages of human *T.b.gambiense* (Hutchinson and Watson, 1962) and *T.b.rhodesiense* infections has been reported (Apted, 1962; de Raadt, Van Hove, Onyango and Kimber, 1965a; de Raadt, Van Hove, Bailey and Kinyanjui, 1965b; Bailey, 1967 and 1968; Onyango, Bailey, Okach, Mwangi and Oganda., 1970; Temu and Mbwabi, 1975; Abaru and Matovu, 1981).

Berenil is administered intramuscularly at 5mg/kg in three doses at one or more day-intervals over a duration of 7-12 days (Abaru and Matovu, 1981). Oral administration with successful outcome has been reported (Bailey, 1968).

The drug is generally well tolerated and toxic side effects are rare except for a slight and persistent albuminuria. Other side effects may include pyrexia, body aches, burning sensations, nausea and or vomiting, weakness and or paralysis of limbs, numbness and itching (Abaru and Matovu, 1981). One isolated case of disorientation and another of coma have been reported (Abaru and Matovu, 1981).

### 1.5.2.2 Late-stage drugs

The development of arsenical drugs was the greatest advance in the treatment of sleeping sickness since the introduction of suramin and tryparsamide in the early 1920s. Friedheim carried out field trials with the first of these drugs, Melarsen, in French West Africa in 1939 (Friedheim, 1949) with very promising results. Other drugs in this group include: melarsen oxide, melarsoprol (Mel B), melarsonyl potassium (Mel W) and antimony derivatives. Of these, only melarsoprol has become firmly and widely established among the drugs of real value in the treatment of sleeping sickness. Melarsen and melarsonyl potassium have proved themselves of benefit in some areas in West Africa. Melarsen oxide was shown to be active against tryparsamide resistant *T.b. gambiense* infections (Van Hoof, 1947; Williamson and Lourie, 1948) at a time when tryparsamide resistance had reached 80% in Congo (Friedheim, 1949). Trials of melarsoprol in treatment of *rhodesiense* sleeping sickness in Tanzania reduced death rates from over 33% to 10% (Apted, 1957).

#### 1.5.2.2.1 Melarsoprol

Melarsoprol was formed from melarsen oxide by the incorporation of dimercaprol (BAL) after melarsen oxide had proved to be too toxic. Melarsoprol contains 18.8% arsenic, and cures both the *rhodesiense* and *gambiense* disease at all stages, even the very advanced stages. A course of injections over 2 to 4 weeks is necessary and even in areas where tryparsamide is effective, the shorter course of treatment gives melarsoprol a great advantage. The improvement is usually rapid, even dramatic, and is often marked by a sense of well-being noticeable within the first few days. In particular patients who are dull or mentally confused, as they often are in the advanced stage of the disease, lose this dullness and become alert and cheerful soon after the start of treatment. Occasionally a patient, particularly one who is in advanced stage of

the disease, may fail to respond to melarsoprol (Apted, 1970).

There is no single recommended treatment regime for melarsoprol despite the fact that it has been in use for over 50 years. Dosage and duration of therapy differ considerably between centres, e.g., the total dose for an adult in Cotê D'Ivoire is 1.62g over a duration of 32 days while 2.16g over 26 days was, until recently, routine practice in Zaire (Van Nieuwenhove, 1992). Progressively increasing doses are administered by some therapists, while maximum daily doses are given straight away by others (WHO, 1986; Van Nieuwenhove, 1992). For instance, the current treatment of both early and late stages of sleeping sickness due to *T.b. gambiense* infection, is a modified form of the dosage scheme proposed by Neujean (1950). For an adult patient weighing 50kg, daily intravenous injections are given of 3.6mg/kg which is equivalent to a daily dose of 180mg. Patients with fewer than 20 cells/mm<sup>3</sup> in the CSF receive one series of daily injections for 4 days. Patients with 21-100 cells/mm<sup>3</sup> receive two series of four injections separated by an interval of 10 days. Those with more than 100 cells/mm<sup>3</sup> receive three series of four injections at 10-day intervals (WHO, 1986).

In many centres, melarsoprol is preceded by a single or multiple injections of the early-stage drugs, suramin or pentamidine along with corticosteroids at 1mg/kg/day in some centres, while in others it is given alone (WHO, 1986; Van Nieuwenhove, 1992).

Trivalent arsenicals like melarsoprol have a high affinity for sulphhydryl groups which form active sites of many enzymes especially kinases. Melarsoprol markedly inhibits pyruvate kinase in intact trypanosomes thus disrupting ATP production and energy generation in African trypanosomes (Flynn and Bowman, 1974). It also combines with trypanothione to form a complex that removes the latter from the system which results in disruption of the trypanosomal protein synthesis (Henderson and Fairlamb, 1987).

Sulphhydryl groups are also involved in the maintenance of secondary

and tertiary structures of proteins, as a result of which side effects to melarsoprol are common and sometimes quite severe. Reactions which may occur, include, fever, chest pain, gripping abdominal pains and subjective sensations of heat and disturbance of smell (Gutteridge, 1985). The most serious side effect is a 5-10% incidence of severe reactive arsenical encephalopathy, that results in neurological damage or death (Hurst, 1959; Gutteridge, 1985; Adams *et al*, 1986; Arroz, 1987). There are three characteristic syndromes of arsenical encephalopathy: status epilepticus with acute cerebral edema; rapidly progressive coma without seizures; and non-fatal cognitive abnormalities without neurological signs (Haller *et al*, 1986).

#### 1.5.2.2.2 Eflornithine

Eflornithine is the most recent drug used for the treatment of the meningoencephalitis, associated with African trypanosomiasis. Eflornithine irreversibly inhibits ornithine decarboxylase (ODC) (Metcalf, Bay, Danzin, Jung, Casara and Vervet, 1978), the key enzyme in the synthesis of the polyamines, including, putrescine, spermidine, and spermine, from ornithine. By inhibiting synthesis of putrescine, eflornithine inhibits differentiation and proliferation of trypanosomes (Beyer, 1978; Bacchi, Nathan, Hunter, McCann and Sjeordsma, 1980; McCann, Bitonti, Bacchi and Clarkson, 1987; Pepin, Milord, Guern and Schechter, 1987; Doua, Boa, Schechter, Miezian, Diali, Sanon, de Raadt, Haegele, Sjeordsma and Konian, 1990), as it does a number of rapidly proliferating cells *in vitro* (Pegg and McCann, 1982; Luk, Goodwin, Marton and Baylin, 1981; Seidenfeld, Gray and Marton, 1981) and in animal tumours *in vivo* (Sunkara, Prakash and Rosenberger, 1982; Sunkara, Prakash, Mayer and Sjeordsma, 1983).

One of the earliest effects ascribed to eflornithine in mammalian cells is an inhibition of protein synthesis (Rudkin, Marmont and Seiler, 1984) which

parallels the depletion of spermidine. These changes are readily prevented by the presence of putrescine or spermidine in addition to eflornithine, i.e., by-passing the enzymic block of ornithine decarboxylase.

Eflornithine was first demonstrated to cure experimental *T.b. brucei* infection by Bacchi *et al* (1980). Subsequent treatment of late-stage trypanosomiasis by the combined use of eflornithine with other compounds has been extremely successful and cures in the experimental mouse model have been reported using eflornithine in combination with bleomycin (Clarkson, Bacchi, Mellow, Nathan, McCann and Sjeordsma, 1983), suramin (Clarkson, Bienen, Bacchi, McCann, Hunter and Sjeordsma, 1984), guanylhydrazones (Jennings, Ulrich and Cerami, 1987), melarsoprol (Jennings, 1988a; 1988b; 1990), the antimonials (Jennings, 1990), 9-deazainosine (Bacchi, Nathan, Clarkson, Bienen, Bitonti, McCann and Sjeordsma, 1987) and Berenil (Bacchi and McCann, 1987; Jennings, 1992).

Successful clinical trials in human *T.b. gambiense* infections were first reported by Van Nieuwenhove, Schechter, de Clercq, Bone, Hurke and Sjeordsma (1985) in the Sudan. Subsequently, eflornithine has been successfully used in treating arseno-resistant late-stage cases of *gambiense* infections in man (Sjeordsma and Schechter, 1984; Schechter and Sjeordsma, 1986; McCann *et al*, 1987; Schechter, Barlow and Sjeordsma, 1987; Pepin *et al*, 1987; Doua *et al*, 1990). It is administered intravenously at a dose rate of 400mg/kg per day for 6 weeks.

Side effects, include, diarrhoea, abdominal pain, vomiting, anaemia, thrombocytopaenia, convulsions, loss of hearing acuity, alopecia, skin eruptions and exacerbations of intercurrent infections. Most of these side effects are reversible upon discontinuation or decreasing eflornithine doses, and convulsions do not recur even after resumption of treatment (Van Nieuwenhove *et al*, 1985; Doua *et al*, 1990; Pepin and Milord, 1991; Pepin, Milord, Meurice, Ethier, Loko and Mpia, 1992).

### 1.5.2.2.3 Nifurtimox

Nifurtimox, a 5-nitrofurans administered orally, has been available for nearly 20 years for the treatment of Chagas' disease, caused by *Trypanosoma cruzi*. There have been a few clinical trials involving nifurtimox in the treatment of *T.b.gambiense*; these were prompted by the demonstration of activity against this parasite in an animal model (Janssens and de Muynck, 1977). These trials have yielded equivocal results. Eighty percent of patients were cured using 15mg/kg/day in three doses for 60 days in both Northern Zaire (Moens, de Wilde and Ngato, 1984) and the Sudan (Van Nieuwenhove and de Clercq, 1989). However using the same dosage for the same duration, cures were obtained in only 31% of the cases in Central Zaire (Pepin, Milord, Mpia, Meurice, Ethier, Degroof and Bruneel, 1989b; Pepin *et al*, 1992). Pepin *et al*, 1992 reported that while doubling the dosage and halving the duration of treatment yielded higher cure rates, the toxic effects outweighed the benefits of this regimen. Studies conducted in the Sudan on arseno-resistant cases of the late-stage *gambiense* sleeping sickness, showed the optimum nifurtimox regimen to be 5mg/kg *per os* (p.o.), three times a day in adults and 6.5mg/kg p.o. three times a day for children for 14 days (maximum 21 days) (Van Nieuwenhove, 1992). The efficacy of this dosage programme was high and there was a considerable reduction in toxicity and mortality (Van Nieuwenhove, 1992). Optimum treatment schedules for the early-stage *gambiense* as well as for *rhodesiense* trypanosomiasis have not yet been defined. Rigorous supervision of drug intake is necessary.

Initial use of Lampit, in man, in combination with trimelarsen gave variable results. However, using a regimen of two daily doses of 50mg/kg for five consecutive days, in combination with single daily doses of 2.5mg/kg trimelarsen produced cures in experimental *T.b.brucei* infections in mice (Jennings, 1991b). Other researchers (Raseroka and Ormerod, 1986), reported

improved cure rates after using high-dose nifurtimox therapy in combination with suramin in a mouse model.

The mode of action of this group of drugs is not well understood. It is thought that 5-nitrofurans interfere with biosynthetic reactions, especially of nucleic acid and protein synthesis (Sims and Gutteridge, 1978; 1979). They interact with DNA causing single- and double-strand breakages. Metabolism of nitrofurans creates superoxide anions such as hydrogen peroxide and since trypanosomes lack catalase, the superoxide anions accumulate to cytotoxic levels in the parasites, killing them (Docampo and Stoppani, 1979; Docampo and Moreno, 1984). Superoxide anions produced by the drug metabolism could interact with DNA and interfere with nucleic acid synthesis.

Nifurtimox is metabolised not only by the parasite but also by the liver, testes and brain of the mammalian host. The drug is not well tolerated and causes side effects. Complications reported by various investigators include: central nervous system toxicity, such as, vertigo, convulsions (which do not recur after resumption of treatment), status epilepticus, psychotic reactions, cerebellar syndrome; peripheral polyneuropathy, anorexia and weight loss; gastro-intestinal discomfort leading to nausea, vomiting, epigastric pain etc; skin rashes, and exacerbation of pre-existing symptoms such as headaches, arthralgia etc (reviewed by Van Nieuwenhove, 1992). The side effects can become quite unpleasant and frightening for the patient, necessitating continuous reassurance and rigorous supervision of drug-intake as prerequisites for successful therapy (Van Nieuwenhove, 1992).

Pepin, *et al* (1992) compared the effects of high-dose nifurtimox and eflornithine on arseno-resistant *T.b. gambiense* patients. They found eflornithine to be of superior therapeutic value since only 10% (at most) patients relapsed following intravenous administration of eflornithine compared to a 36% relapse rate with nifurtimox. Furthermore, the toxicity associated with eflornithine therapy was more acceptable compared to that of high-dose nifurtimox therapy.

They concluded that high-dose nifurtimox should be used only for the rare patients who had relapse after both melarsoprol and eflornithine, or in arseno-resistance cases if eflornithine was unavailable.

### **1.5.3 Supportive therapy: immunomodulation**

The CNS lesion found in human African trypanosomiasis is an immune-cellular reaction in the meninges, Virchow Robin spaces and, in the late stages, the neuropil (Adams *et al*, 1986; Haller *et al*, 1986; de Raadt, 1984). The reactive encephalopathy that occurs in up to 10% of patients following melarsoprol therapy is an exacerbation of this cellular reaction with inconsistent additional features such as hypoxic changes and haemorrhages (Robertson, 1963). Ideally, some method of preventing the cellular infiltrate from developing or reversing an already initiated cellular infiltration would greatly enhance management of late stages of human African trypanosomiasis. To this end various anti-inflammatory drugs have been used in the chronic trypanosome infections in man and murine models, including, corticosteroids, and azathioprine (Arroz, 1987; Jennings *et al*, 1989; Hunter *et al*, 1992c).

#### **1.5.3.1 Corticosteroids**

Several investigators (Foulkes, 1975; Arroz 1987; Pepin, Milord, Guern, Mpia, Ethier and Mansisa, 1989a) have reported on the use of corticosteroids in treatment of reactive arsenical encephalitis (RAE) in late-stage sleeping sickness. While Arroz (1987) reported that corticosteroids had no beneficial value in the prevention or treatment of RAE, other investigators reported that prednisolone reduced the incidence of RAE but had no effect on the fatal outcome once the encephalopathy had developed (Pepin *et al*, 1989a). To date, the value of corticosteroids, in this respect, remains uncertain.

### 1.5.3.2 Azathioprine

Azathioprine is a non-steroidal anti-inflammatory drug, that is used in prevention of heterograft rejection. It has been reported to inhibit renal homograft rejections in dogs (Calne and Murray, 1961; Zukosi, 1965), prolong renal allograft survival in Rhesus monkeys (Dicke, Marquet, Heyshek and Balner, 1971), and to inhibit rejection of full thickness corneal xenograft in rabbits (Leibowitz and Elliott, 1966). It has also been shown to inhibit the secondary phase of adjuvant arthritis in a mouse model of arthritis (Whittington, 1970; Perper, Alvarez, Colombo and Schroder, 1971), and the development of experimental allergic encephalitis (EAE) in rats, a model of multiple sclerosis (Rosenthale, Dakto, Kassarich and Schneider, 1969; Babington and Wedeking, 1971).

Azathioprine is a 6-substituted purine, a structural analogue of adenine and hypoxanthine. It is readily cleaved into the active 6-mercaptopurine (6-MP) in blood and tissues (Chalmers, Knight and Atkinson, 1967). The process is non-enzymatic and takes place mainly in erythrocytes but the liver is necessary for further metabolism of the cleavage product, 6-MP (de Miranda, Beacham, Creagh and Elion, 1973). Azathioprine can also be split by glutathione and sulphhydryl groups in proteins into methylnitrothioimidazole (Chalmers *et al*, 1967; Elion, 1972; de Miranda *et al*, 1973); and into 6-MP and amino-1-methyl-4-nitroimidazole (de Miranda, *et al*, 1973).

6-MP is a competitive inhibitor of hypoxanthine guanine phosphoribosyl transferase. It competes with hypoxanthine inhibiting purine biosynthesis. As an analogue of inosine monophosphate (IMP), 6-MP inhibits a variety of purine interconversions (Elion, 1967), the most sensitive being the conversion of IMP to succinyl adenine monophosphate (AMP), the conversion of IMP to xanthine monophosphate (XMP) and the synthesis of phosphoribosylamine (Salser, Hutchinson and Balis, 1960; Elion 1967).

## 1.6 POSSIBLE PATHOGENIC MECHANISMS

The pathogenesis of African trypanosomiasis, particularly as it relates to the development of the CNS disease, is poorly understood. Numerous hypotheses have been proposed but few firm data are available upon which to base a cohesive explanation of the mechanisms that result in trypanosomal CNS disease. Many descriptive accounts on the lesions in trypanosome-infected man have been documented (Calwell, 1937; Manuelidis *et al*, 1965; Poltera *et al*, 1976; Adams *et al*, 1986; Haller *et al*, 1986; Cegielski and Durack, 1991), but the amount of information on the mechanisms involved is limited.

The outstanding pathological effects of African trypanosomes are anaemia, lymphoid cell proliferation and immunodepression, circulatory disturbances associated with increased vascular permeability and severe tissue damage (Murray, Morrison, Emery, Akol, Masake and Mooloo, 1979; Greenwood and Whittle, 1980; Murray and Morrison, 1980; Vickerman and Barry, 1982). There are at least three ways in which trypanosomes could damage their host: production of toxins, induction of harmful metabolic changes in the host, or induction of an immunopathological reaction.

### 1.6.1 Toxin production

Several investigators have reported the existence of biologically active substances of trypanosome origin, including, haemolysins, inflammatory and permeability factors, complement activating factors, platelet aggregating factor, immunosuppressive factors and polyclonal B cell mitogens (Seed, 1969; Davis *et al*, 1974; Woo and Kobayashi, 1975; Morrison, Murray and Sayer, 1979; Tizard, Nielsen, Seed and Hall, 1978; Murray, 1979). In most instances, however, these factors have been detected *in vitro*. 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. It is possible that these factors act by inducing an immunopathological reaction.

Esievo and Saror (1983) suggested that leukopaenia could result from plasma neuraminidase released by trypanosomes (Esievo, 1979), which cleaves sialic acid from leukocytes, directing them from the lymph nodes and spleen to the liver for destruction, which occurs in a similar manner to that reported to occur with the influenza virus neuraminidase (Woodruff and Woodruff, 1976). Trypanosomes have also been reported to release a peptidase which could cause red cell damage (Knowles, Black and Whitelaw, 1987).

Phospholipase A<sub>1</sub> and lysophospholipase 1, have been isolated from suspensions of a number of trypanosome species, including, *T.b. brucei*, *T. congolense*, *T. lewisi* and *T. theileri* with *T.b. brucei* suspensions showing the highest levels (Mellors, 1985). Since phospholipases are frequently associated with membrane remodelling, the primary role of *T.b. brucei* phospholipases could be in the development of pleomorphs such as stumpy forms from the slender forms of trypanosomes. Lower levels of phospholipase A<sub>1</sub> in stercorarian and hematic trypanosomes suggest a role for the enzyme in crossing the reticuloendothelial barrier of the mammalian host or the gut wall of the insect vector (Mellors, 1985). Indeed, high levels of phospholipase A<sub>1</sub>, have been reported to occur in tissue fluids during peak parasitaemia in *T.b. brucei*-infected rabbits, and to rise and fall with the parasitaemic waves (Sage, Hambrey, Werchola, Mellors and Tizard, 1981).

High levels of phospholipase A<sub>1</sub> leads to generation of membrane-active products from phospholipids such as free fatty acids, which are immunosuppressive *in vivo* and *in vitro* (Meade and Mertin, 1975), making hosts susceptible to secondary infections. This might contribute to the generalised immunosuppression that occurs in trypanosomiasis. Direct attack on the host's membrane lipids by trypanosomal phospholipases may be pathologically important. In addition, phospholipases could cause the release of

low density lipids and very low density lipids leading to cachexia and hyperlipidaemia observed in trypanosome infections in man and animals (Dixon, 1967; Diehl and Risby; 1974, Greenwood and Whittle, 1980; Ndung'u, 1990). In addition, phospholipases have been reported to induce prostaglandin D<sub>2</sub> and cytokine production in the CNS (Pentreath, Rees, Owolabi, Philip and Doua, 1990; Fontana, Kristensson, Dubs, Gemsa and Weber, 1982) leading to slow wave sleep and CNS inflammation.

### **1.6.2 Induction of harmful metabolic changes**

In infections of experimental animals, trypanosomes may be present in sufficiently large numbers to produce biochemical changes in the host. Large amounts of lactic acid and pyruvate are produced (Goodwin, 1974) and it is possible that these metabolites could damage small blood vessels and that they could interfere with cardiac and cerebral function. African trypanosomes are capable of metabolising aromatic amino acids such as tryptophan and phenylalanine, to a series of potentially toxic compounds, including, indole-ethanol and phenylpyruvate (Seed and Sechelski, 1977). Indole-ethanol induces a sleep-like state and alters body temperature when administered at pharmacological levels in rabbits (Seed and Sechelski, 1977), and is immunosuppressive (Seed, 1980). Indole-ethanol has also been shown to alter membrane properties leading to osmotic fragility and lysis (Seed, Hall and Sechelski, 1982); to cross the blood-brain barrier and to alter the brain metabolism (Seed, Hall and Price, 1983). Phenylpyruvate has a number of inhibitory effects, including, inhibition of mitochondrial function (Patel, Booth and Clark, 1977), gluconeogenesis and proteolytic activity (Lane and Neuhoff, 1980). Elevated levels of these metabolites have been reported to occur in laboratory animals infected with African trypanosomes, and might account for some of the pathology, such as anaemia and emaciation. These metabolic

pathways might deplete the precursor aromatic amino acids or their biologically active metabolites, including, hormones, neurotransmitters and protein precursors (Seed *et al*, 1983). In addition, in acute infections with very high parasitaemia, trypanosome numbers in the host may reach levels that would compete for glucose with the mammalian host causing acidosis. Indeed, lactic acidosis has been reported to occur in terminal stages of *gambiense* sleeping sickness (Greenwood and Whittle, 1980).

### **1.6.3 Immunopathogenesis**

The immune reaction, which at the outset is beneficial to the host, may actually have adverse effects if it is sustained and intensified. It is highly likely that this is what happens in trypanosome infections. Indeed, granular deposits of immunoglobulins and trypanosome antigen were reported to occur in between muscle fibres, in the heart and skeletal muscle sections, of *T.b. brucei*-infected mice (Galvao-Castro, Hochmann and Lambert, 1978). In addition, Galvao-Castro and colleagues (1978) demonstrated that the severity of lymphocyte/plasma cell infiltrations into the tissues, was related quantitatively to the amount of immunoglobulin deposits, and that such infiltrates were reduced in the muscles of immune deprived mice. Furthermore, the characteristic inflammatory cell-infiltration into tissues of trypanosomiasis patients indicates that some form of immunopathological reaction is involved in the pathogenesis of sleeping sickness (Ormerod, 1970; Greenwood and Whittle, 1980; Poltera, 1985; Cegielski and Durack 1991). The nature of this response is poorly understood.

#### **1.6.3.1 Changes in the lymphoid organs**

The clinical features of the African trypanosomiases can in large measure be attributed to the gross proliferative response of B lymphocytes, first in the lymphoid system itself and later in other tissues and organs, including the

CNS. Thus, the enlargement of the lymph nodes and spleen largely result from an increase of the B lymphocytes in the germinal centres and the lymphoid follicles (Ormerod, 1970; Murray *et al*, 1974a; Mansfield and Bagasra, 1978; Morrison and Murray, 1979; Greenwood and Whittle, 1980; Morrison, Murray and Bovell, 1981a; Morrison *et al*, 1981b; Morrison, Murray and Hinson, 1982). The T-cell dependent zones (the paracortical zones of the lymph nodes and the periarteriolar regions of the spleen) become depleted of small lymphocytes and heavily infiltrated with plasma cells and macrophages (Ormerod, 1970; Murray *et al*, 1974a; 1974b; Morrison and Murray, 1979; Morrison *et al*, 1981a; 1981c; 1982). Immunofluorescence studies have shown that B, T and null cells are stimulated to divide but that the T-cell response is short lived (Mayor-Withey, Clayton, Roelants and Askonas, 1978; Morrison and Murray, 1979). It has been suggested that the trypanosome may act as polyclonal B cell mitogen (Urquhart, Murray, Murray, Jennings and Bate, 1973; Hudson *et al*, 1976) and it has been shown that some trypanosome fractions are mitogenic (Assoku, Hazzlet and Tizard, 1979).

The pathogenesis of African trypanosomiasis in man is dominated, as in other species, by the trypanosome's ability to undergo antigenic variation in the face of the host's immune response (Onyango, de Raadt, Cunningham and Van Hove, 1965; Murray *et al*, 1979; Hajduk, England and Smith, 1990). Thus, the disease is characterised by progressive waves of parasitaemia, each wave consisting of a different antigenic variant of trypanosomes (Koten and de Raadt, 1969; Hajduk *et al*, 1990). Waves of immunoglobulins corresponding to each antigenically new population of trypanosomes, are generated resulting in massive hypergammaglobulinaemia, particularly IgM (Koten and de Raadt, 1969; Hajduk *et al*, 1990). Although the levels of IgG are not as elevated as those of IgM, Nielsen, Shephard, Holmes and Tizard (1978) showed that there is a higher catabolic rate for IgG than for IgM suggesting that IgG production is

elevated too. Evidence from laboratory animal infections indicates that much of the IgG produced is not directed against the trypanosome (Freeman, Hudson, Longstaffe and Terry, 1973; Corsini, Clayton, Askonas and Ogilvie, 1977) but contains heterophile antibodies, rheumatoid factor-like substances and a range of auto-antibodies (Mackenzie and Boreham, 1974; Murray *et al*, 1974b).

### **1.6.3.2 Immune complex mediated pathology**

Raised soluble immune complex levels are found in the sera of both experimentally infected animals and sleeping sickness patients (Fruit, Santoro, Afchain, Durallet and Capron, 1977; Galvao-Castro, Hochmann and Lambert, 1978; Whittle, Mohamed and Greenwood, 1980; Greenwood and Whittle, 1980; Poltera, 1985). The higher levels of immune complexes, found in advanced-stage of infection, are attributable to repeated destruction of the trypanosome populations by host antibody and the shedding of antigen by the live trypanosomes into an antibody laden environment.

Immune complex-deposition in tissues invaded by trypanosomes has been reported to occur in both rodents (Murray, 1974; Murray, Lambert and Morrison, 1975; Poltera, 1980) and primates experimentally infected with African trypanosomes (Poltera, 1980). Such immune-complexes are thought to play an important part in the pathogenicity of such experimental infections. Deposition of immune complexes at the sites of invasion by trypanosomes might explain many of the clinical, laboratory and pathological features of sleeping sickness, such as damage to small vessels and anaemia, the latter resulting from cytoadherence of the immune complexes and subsequent erythrophagocytosis (Murray and Morrison, 1980; Morrison *et al*, 1979). The resultant anaemia might lead to tissue anoxia and subsequent tissue lesions.

Immune complexes are capable of activating the Hageman factor and therefore the complement and kallikrein-kinin systems, causing the release of kinins and other autacoids, such as, histamines, 5-hydroxytryptamine and

catecholamines, leading to increased capillary permeability and resultant tissue oedema (Goodwin and Richards, 1960; Boreham and Goodwin, 1970; Boreham, 1968; 1970). This oedema, together with the infiltrating cells might disrupt tissues especially in the skeletal muscle, the myocardium and the brain. In this way the separation of the blood vessels of the choroid plexus from the ependymal lining might impair the secretion of CSF, while the separation of the Purkinje cells in the heart from myocardium interferes with the conducting system of the heart (Morrison *et al*, 1979; Vickerman and Barry, 1982). Immune complexes and their related lesions did not occur in tissues of T cell-deficient, trypanosome-infected mice (Galvao-Castro *et al*, 1978; Poltera, Hochmann, Rudin and Lambert, 1980a; Poltera, Hochmann and Lambert, 1980b). This implies a helper role for T cells in the synthesis of the relevant antibodies and suggests that direct damage by trypanosomes and their products do not play a major role in the pathogenesis of trypanosomiasis.

Pruritis and urticaria are occasional features of sleeping sickness and might result from the immune complex-mediated release of autocoids, such as histamines (Boreham, 1985), or from type 1 immediate hypersensitivity to trypanosome products (Greenwood and Whittle, 1980). Eosinophilia, a frequent accompaniment of immediate hypersensitivity reactions, is not a feature of untreated sleeping sickness, but eosinophil counts may rise following the start of trypanocidal therapy (de Raadt, 1974), perhaps as part of the response to dying or dead trypanosomes.

It has been suggested that the interaction of immunoglobulins with trypanosomes or trypanosome antigens within the tissues is a more important mechanism for tissue disruption, than the deposition of immune complexes from the blood circulation (Murray *et al*, 1979; Murray and Morrison, 1980). The antibodies involved may enter the tissues from the circulation or may be synthesised locally by the infiltrating plasma cells (Lambert *et al*, 1981). The

presence of immune complexes in the kidneys of *T.b. brucei*-infected rabbits (Goodwin and Guy, 1973), might explain the renal insufficiency and the progressive proteinuria found in such rabbits (Itazi and Enyaru, 1973). Increased vascular permeability might also explain the increased protein levels in the CSF of sleeping sickness patients (de Raadt, 1984; Adams *et al*, 1986; Mbulamberi, 1989) and of experimentally *T.b. rhodesiense*-infected monkeys (Fink, Sayer and Schmidt, 1983).

The role of immune complexes in mediating the CNS inflammatory process is controversial. Thus, Greenwood and Whittle (1980) were unable to detect immune complexes in the CSF of *gambiense* sleeping sickness patients, suggesting that immune complexes do not play an important part in producing brain damage. In contrast, Lambert *et al* (1981) demonstrated intracranial immunoglobulin synthesis, and the presence of immune complexes in the CSF of *gambiense* sleeping sickness patients. The deposition complement factor 3 (C3) and immunoglobulins in the CNS of trypanosome infected animals (Poltera *et al*, 1980a; 1980b) suggest immune complex mediated activation of the complement system.

Arthus-type reactions, featuring polymorphonuclear leukocytes and necrotising vasculitis resulting from local immune complex formation in tissues have been reported to occur in dogs, to be scanty in rabbits and are absent in sleeping sickness patients (Goodwin, 1974; Morrison *et al*, 1979; Greenwood and Whittle, 1980). The tissue invasiveness of the *Brucei* group of trypanosomes would make it likely that a hypersensitivity mechanism of this type may be operative in human trypanosomiasis. However, the lack of polymorphonuclear leukocytes and Arthus-type reaction in tissues, and of serum sickness-type reactions in sleeping sickness patients, makes the role of locally-formed immune complexes in the pathogenesis of HAT unclear.

### 1.6.3.3 Autoimmunity

Autoantibody production is a prominent feature of African trypanosomiasis and tissue damage might be produced in this way (Poltera, 1980). It has been suggested that autoantibodies directed at trypanosome antigen bound to host red blood cells might be responsible for the haemolytic anaemia of *T.b.brucei* infections of mice (Woo and Kobayashi, 1975). It has been proposed that attachment of parasite antigens onto brain cells might prime the host immune system to attack these cells (Pepin and Milord, 1991). Indeed, Rifkin and Landsberger (1990) showed that trypanosome VSG is capable of inserting itself into cell membranes. The presence of autoantibodies to myelin basic protein in sera of *gambiense* sleeping sickness patients has been reported (Asonganyi, Lando and Ngu, 1989). However, de Raadt (1974) failed to detect anti-neuronal antibodies in patients with *rhodesiense* sleeping sickness. Autoantibodies to myelin basic protein, galactocerebrosides, gangliosides and double stranded DNA have been reported to be elevated during *T.b. brucei* infections in mice and to correlate with the severity of CNS pathology (Hunter, Jennings, Tierney, Murray and Kennedy, 1992c). However, the rapidity and effectiveness with which health is restored when the parasite antigen is removed after chemotherapy, suggests that autoimmunity does not play a very important role in the pathogenesis of trypanosomiasis. It is possible that the presence of autoantibodies is a result and not a cause of tissue damage.

### 1.6.3.4 Immunosuppression

Immunosuppression is an important feature of the pathology of African trypanosomiasis in man (Greenwood, Whittle and Molyneux, 1973), and laboratory animals (Goodwin, 1970; Goodwin, Green, Guy and Voller, 1972; Murray *et al*, 1974b; Mansfield and Bagasra, 1978; Hudson and Terry, 1979). Thus, the host is rendered more susceptible to secondary infections and gives

diminished responses to viral and bacterial vaccines in man (Greenwood *et al*, 1973) and animals (Scott, Pegram, Holmes, Pay, Knight, Jennings and Urquhart, 1977). For instance, the cellular response of *gambiense* sleeping sickness patients to purified protein derivative of candida and streptococcal antigens, and the humoral response to *Salmonella typhi* vaccine was found to be depressed (Greenwood *et al*, 1973).

Evidence of immunosuppression in experimental animals came initially from observations on impaired antibody response to sheep red blood cells in *T.b. brucei*-infected mice and rabbits (Goodwin, 1970; Goodwin *et al*, 1972; Longstaffe, Freeman and Hudson, 1973). Suppressed B-lymphocyte responses to other helper T cell-dependent antigens, such as bovine serum albumin, and T cell-independent antigens, such as bacterial lipopolysaccharide, have also been noted (Ackerman and Seed, 1976; Murray *et al*, 1974b). It has also been reported that the antibody response to the trypanosome itself was depressed (Hudson and Terry, 1979). The treatment of trypanosome infection rapidly restored immunocompetence and enables the host to mount a full secondary response (Freeman, Hudson, Longstaffe and Terry, 1973; Murray *et al*, 1974b; Wellhausen and Mansfield, 1980). This suggested that although trypanosome infection blocks the expression of a secondary immune response, immunological memory is preserved. However, this immunological memory may become exhausted in *T.b. brucei*-infected mice as is shown by the inability of irradiated recipient mice to mount a secondary response following transfer of sensitised spleen cells from infected mice (Askonas, Corsini, Clayton and Ogilvie, 1979).

Cell mediated responses may also be depressed by trypanosome infections. For example, the T cell responses induced by oxazolone treatment of the skin of mice was found to be depressed as indicated by measurement of ear thickness (Urquhart *et al*, 1973; Ackerman and Seed, 1976), and by cell incorporation of <sup>125</sup>I-labelled iododeoxyuridine in local lymph nodes (Urquhart *et al*, 1973).

The stimulus to B lymphocytes in patients with sleeping sickness is a massive one and results in disordered immunoglobulin synthesis with the appearance of incomplete IgM molecules and free light chains (Greenwood and Whittle, 1980). It is possible that when this stimulus is sustained, the B lymphoid system becomes exhausted and that this exhaustion contributes to the immunosuppression associated with the infection (Greenwood and Whittle, 1980). It is also possible that, immunosuppression may be due to decreased immunoglobulin synthesis and release, but also due to increased immunoglobulin catabolism, as has been found in *T. congolense*-infected calves (Nielsen *et al*, 1978).

Spleen cells from *T.b. brucei*-infected mice suppress the ability of normal mouse spleen cells to respond to mitogenic stimuli *in vitro* (Eardley and Jayawardena, 1977; Pearson, Roelants, Pinder, Lundin, and Mayor-Withey, 1979; Wellhausen and Mansfield, 1979). Thus, removal of T cells from a culture of spleen cells, from infected mice, restored the response of B cells to lipopolysaccharide. In addition, macrophages from an early mouse infection also depress the proliferative response of normal spleen cells (Corsini *et al*, 1977; Wellhausen and Mansfield, 1979). These observations indicate the presence of two populations of suppressor cells in the spleen of infected mice. Eardley and Jayawardena (1977) suggested that non-specific suppressor T cells are stimulated directly by trypanosomes to produce suppressive factors that act through macrophages. Macrophages seem to play a central role in the development of immunosuppression in murine models. Borowy, Stenberg, Schreiber, Nonnengasser and Overath (1990) showed that the transfer of peritoneal macrophages from infected to uninfected mice produced immunosuppression in recipient mice.

Faulty processing of the trypanosome antigens and their inadequate presentation with the major histocompatibility complex (MHC) class II products

(Ia antigen presentation), and altered secretion of the macrophage-derived immunoregulatory factors are possible means of immunosuppression (Mansfield and Bagasra, 1978; Bagasra, Schell and Le Frock, 1981; Borowy *et al.*, 1990). For instance, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), superoxide anion (O<sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) secretion are increased at the height of parasitaemia (Askonas, 1985). The secretion of interleukin 1 (IL-1) by macrophages from *T.b. brucei*-infected mice is increased and is accompanied by increased prostaglandin synthesis (Sileghem, Darji, Hamers and De Beetselier, 1989a). Production of IL-2, a cytokine responsible for T-cell expansion, is severely depressed by PG-producing macrophages. In addition, the expression of IL-2 receptors on CD4<sup>+</sup> and CD8<sup>+</sup> cells is blocked by PG/H<sub>2</sub>O<sub>2</sub>-independent suppressive mechanisms, without influence on IL-2 production during trypanosome infections of mice (Sileghem, Darji, Hamers and De Beetselier, 1989b). Depletion of CD8<sup>+</sup> cells, which are responsible for the marked increase in interferon gamma (IFN- $\gamma$ ) production during trypanosome infections, has been shown to suppress parasite growth and increase survival of infected mice (Bakheit, Olsson, Van der Meide and Kristensson, 1990). IFN- $\gamma$  is a potent activator of macrophages but it can also inhibit macrophage-mediated T-cell proliferation. Thus, PGs and IFN- $\gamma$  may be soluble factors that trigger macrophages to have immunosuppressive effects on target cells.

Tumour necrosis factor (TNF), a cytokine produced by endotoxin-stimulated macrophages, increases liver triglyceride clearance and anabolism causing both hyperlipidaemia and cachexia, which are common in trypanosomal infection. This has been reported in *T.b. rhodesiense* infected rats by Dixon (1967), *T.b. brucei* infected dogs (Ndung'u, 1990) and rabbits (Goodwin and Guy, 1973; Rouzer and Cerami, 1980) and in *T.b. gambiense* infected rabbits (Diehl and Risby, 1974). Similar observations were made in human *T.b. gambiense* patients by Huet, Lemesre, Grard, Noireau, Boutignon, Dieu, Jannin and Degand (1990). It has been found that human very low density lipids

(VLDL) and low density lipids are highly immunoregulatory *in vivo* (Curtis, Deheer and Edgington, 1977) and *in vitro* (Chisari, 1977; Morse, White and Goodman, 1977). Increase in serum levels of these lipids during trypanosome infections may contribute to the immunosuppressed state of infected man and animals.

The progressive loss of antigen-presenting macrophages from the spleen and later from the lymph nodes and peritoneal cavity of *T.b. rhodesiense* infected mice raises the possibility that antigenic competition is responsible for immunosuppression (Bagasra *et al*, 1981). One explanation of such competition is that it takes place at the surface of antigen-presenting macrophages; if these macrophages are reduced in number, in a trypanosome-laden host, then antigenic competition occurs leading to defective response.

#### **1.6.3.5 Immunopathogenesis of the central nervous system lesions**

The pathology in the nervous system is further complicated by the special immune properties of this organ, the blood-brain and blood-cerebrospinal fluid (CSF) barriers and the lack of trypanosome nutrients in the CNS parenchyma. Precisely how the parasites enter the brain parenchyma is not known. Invasion of the CNS by trypanosomes is followed by infiltration of the choroid plexus, the meninges and the perivascular spaces by mononuclear inflammatory cells, including, lymphocytes, plasma cells and macrophages, and the proliferation of astrocytes and microglia in the brain parenchyma (Ormerod, 1970; Poltera, 1985; Haller *et al*, 1986; Adams *et al*, 1986). Parasites may penetrate certain regions of the brain where the blood-brain barrier is incomplete with the resultant induction of MHC class I and class II antigens (Schultzberg, Ambatsis, Samuelsson, Kristensson and Van Meirvenne, 1988; Schultzberg, Olsson, Samuelsson, Maehlen and Kristensson, 1989). MHC antigen expression may be triggered by IFN- $\gamma$  produced by infiltrating CD8<sup>+</sup>

cells (Bakheit *et al*, 1990). The affected areas are involved in the control of sleep and may explain the signs that give sleeping sickness its name. Several immune response modifiers are important in the control of normal sleep and its increase in infectious diseases and fever. IL-1 is somnogenic and is thought to be linked in the regulatory pathways to produce further somnogenic agents, notably prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) (Hayaishi, 1989; Krueger, 1990). A significant increase of PGD<sub>2</sub> in the CSF of *T.b. gambiense*-infected patients has been reported (Pentreath *et al*, 1990).

Recent work has focused attention on the role of astrocytes in HAT (Pentreath, 1989). These cells are potent producers of PGD<sub>2</sub>, IL-1 $\alpha$ , IL-1 $\beta$  and TNF, when stimulated by various factors, including phospholipases, lipopolysaccharide and neurotropic viruses (Fontana *et al*, 1982; Fierz and Fontana, 1986; Murphy, Pearce, Jeremy and Dandona, 1988). There is a concentration of PGD<sub>2</sub> receptors in the preoptic area, which is a centre for sleep. Therefore, elevated levels of PGD<sub>2</sub> in the CNS may cause some of the neurological signs of late-stage trypanosomiasis, such as, somnolence and headache. IL-1 and TNF have the capability of mediating meningeal inflammatory processes (Ramilo, Saez-Llorens. Mertsola, Jafari, Olsen, Hansen, Yoshinaga, Ohkawara, Naruchi and McCracken, 1990; Saukkonen, Sande, Cioffe, Wolpe, Sherry, Cerami and Tuomanen, 1990). Although other cells, such as the microglia and endothelial cells, are also capable of producing such cytokines in the CNS, experimental *T.b. brucei* infections in mice showed a positive correlation between astrocyte activation and the production of cytokines, including, IL-1 and TNF (Hunter, Gow, Kennedy, Jennings and Murray, 1991).

It would appear that the lesions produced in the CNS by the African trypanosomes and the clinical signs associated with these lesions seem to have their basis in the infiltrating inflammatory cells, the intrinsic brain immune auxiliary cells, including, astrocytes and microglia, and the mediators

produced by these cells. How the trypanosomes invade the central nervous system and what attracts B lymphocytes to sites invaded by trypanosomes are unknown; and how infiltration with B cells and plasma cells might impair the cerebral function is a matter of speculation. It is possible that the answers to these questions lie in the cells constituting the CNS lesion and the mediators produced by them, including cytokines (discussed below). A better understanding of pathogenesis might therefore pave the way to better methods of disease management.

#### **1.6.4 The role of cytokines in disease**

Cells communicate with each other not only by cell to cell contact but also via elaboration of soluble mediators or cytokines. Cytokines are critical in the regulation of immune responses, by providing amplification and differentiation signals for the generation of the effector phase of immune responses, and by interacting with each other to down regulate immune responses (Llorente, Crevon, de France, Banchereau and Galanaud, 1989; Moore, Viera, Florentino, Trounstein, Khan and Mossman, 1990). In contrast, the absence of cytokines following antigenic stimulation has been reported to be critical in the induction of tolerance (Nossal, 1989; Malkovsky and Medawar, 1984).

Haemostasis, inflammation and immunity involve close interaction between immunocompetent cells and the vascular endothelium. Thus, immune and endothelial cells are both capable of synthesizing and secreting cytokines following stimulation with chemical and infectious agents. Cytokines produced by endothelial cells are mediators of complex bidirectional interactions between leukocytes and vascular cells, including, the endothelial cells themselves. Endothelial cells also produce polypeptide mediators that regulate haematopoiesis, differentiation and proliferation of T and B cells, and extravasation of

leukocytes, all of which are critical processes in inflammation (Montovani and Dejana, 1989).

It is likely that infectious agents that invade the nervous system from the systemic circulatory system do so by activation of the systemic immune cells and endothelial cells, and probably the endothelium of brain blood vessels. The invading organisms may then utilize the resultant effects to gain entry into the CNS. For this reason, cytokines known to affect the endothelial cell function as well as the immune cells are considered below.

#### **1.6.4.1 Interleukin 1**

Interleukin 1 (IL-1) designates two proteins, IL-1 $\alpha$  and IL-1 $\beta$ , which are products of two distinct genes, but which recognise the same cell surface receptors. IL-1 $\alpha$  and IL-1 $\beta$  are structurally related polypeptides that show approximately 25% homology at the amino acid level (Oppenheim, Kovacs, Matsushima and Durum, 1986). Both are secreted proteins with the IL-1 $\alpha$  precursor showing full biological activity while IL-1 $\beta$  precursor shows only partial biological activity (Rosenwasser, Webb, Clark, Irie, Chang, Dinarello, Gehrke, Wolff, Rich and Auron, 1986; Mosley, Urdal, Prickett, Larsen, Cosman, Conlon, Gillis and Dower, 1987; Jobling, Auron, Gurka, Webb, McDonald, Rosenwasser and Gehrke 1988; Hazuda, Strickler, Kueppers, Simon and Young, 1990)

With the exception of skin keratinocytes, some epithelial cells and certain cells of the CNS, IL-1 is not produced by cells of healthy individuals (Dinarello, 1992a; 1992b). However, in response to stimuli such as those produced by inflammatory agents, infections or microbial endotoxin, a dramatic increase in the production of IL-1 by various cells, occurs (Dinarello, 1991; 1992a; 1992b; Dinarello and Wolff, 1993). IL-1 is primarily a product of activated monocytes and macrophages but has been shown to be produced by most nucleated cells and exerts its effects through specific receptors. IL-1

profoundly alters the anti-thrombotic properties of endothelial cells, inducing tissue-type procoagulant activity by augmenting production of an inhibitor of plasminogen activator and decreasing the tissue type plasminogen activator (Bevilacqua, Schleef, Gimbrone and Loskutoff, 1986b; Nachman, Hajjar, Silverstein and Dinarello, 1986; Emeis and Kooistra, 1986; Gramse, Brevario, Pintucci, Millet, Dejana, Van Damme, Donati and Mussoni, 1986). IL-1 plays a role in leukocyte extravasation indirectly by inducing the production of chemotactic cytokines by endothelial cells and the expression of intercellular adhesion molecule 1 (ICAM-1) and endothelial leukocyte adhesion molecule 1 (ELAM-1) on endothelial cells (Dustin and Springer, 1988; Bevilacqua, Pober, Wheeler, Cotran and Gimbrone, 1985; Bevilacqua, Pober, Majeau, Fiers, Cotran and Gimbrone, 1986a; Hertz, McFarlin and Waksman, 1990). In addition, IL-1 triggers lymphokine release by T cells, co-stimulates differentiation and proliferation of B-cells; augments natural killer (NK) mediated cytotoxicity; induces chemotaxis, degranulation and release of neutrophils from bone marrow; prostaglandin (PG) release, chemotaxis, and tumor cell lysis by macrophages; acute phase protein secretion by hepatocytes, and fever. IL-1 is also a polyclonal activator of lymphocytes causing proliferation of pluripotential progenitors in bone marrow by inducing production of colony stimulating factors.

These reported biological effects of IL-1 range from inducing specific cell type responses to targeting entire systems. Although normal production of IL-1 is obviously critical to mediation of host responses to injury and infection, inappropriate or prolonged production of IL-1 is implicated in the production of a variety of pathological conditions such as inflammatory or autoimmune diseases (Dinarello, 1991; 1992a; 1992b; Dinarello and Wolff, 1993). Techniques for controlling the synthesis of IL-1 will obviously have therapeutic implications for the treatment of a variety of diseases.

#### 1.6.4.2 Tumor necrosis factor

Tumor necrosis factor (TNF) occurs in two forms: TNF $\alpha$  also known as cachectin which is produced primarily by macrophages (Beutler, Greenwald, Hulmes, Chang, Pan, Mathison, Ulevitch and Cerami, 1985a), and TNF $\beta$  also known as lymphotoxin, produced by activated T lymphocytes (Ruddle and Waksman, 1968). Both forms have identical properties and exert their effects by binding to the same cell surface receptors. There are two distinct forms of receptor types, each of which can bind either TNF $\alpha$  or TNF $\beta$  with high affinity (Smith, Davis, Anderson, Solam, Beckmann, Jerzy, Dower, Cosman and Goodwin, 1990; Loetscher, Pan, Lahm, Gentz, Brockhaus, Tabuchi and Lesslauer, 1990). Virtually all cells express one or both of these receptors (Dembic, Loetscher, Gubler, Pan, Lahm, Gentz, Brockhaus and Lesslauer, 1990). There is no evidence that interaction between the two receptor types is necessary for signal transduction (Smith *et al*, 1990; Loetscher *et al*, 1990; Engelmann, Holtmann, Brackebusch, Avni, Sarov, Nophar, Hadas, Leitner and Wallach, 1990).

TNFs are capable of producing a wide range of effects attributable to the ubiquity of their receptors, their ability to activate multiple signal transduction pathways, and to their ability to induce or suppress the expression of a vast number of genes; including those for growth factors and cytokines, transcription factors, receptors, inflammatory mediators and acute phase proteins (Vilcek and Lee, 1991). For instance, TNF acts on endothelial cells initiating expression of adhesion structures for leukocytes (Gamble, Harlan, Klebanoff and Vadas, 1985), and production of chemotactic cytokines and colony stimulating factors (Seelentag, Mermoud, Montesano and Vassalli, 1987). It also stimulates prostacyclin (PG $_{12}$ ) production (Kawakami, Ishibashi, Ogawa, Murase, Takaku and Shibata, 1986), procoagulant activity (Bevilacqua *et al*, 1986a), tissue type plasminogen activator (Van Hinsberg, Kooistra, Van den Berg, Princen, Fiers

and Emeis, 1988), platelet activation factor (Camussi, Bussolino, Salvidio and Baglioni, 1987) and decreases the thrombomodulin-protein C anticoagulation pathways (Conway and Rosenberg, 1988; Moore, Emson and Emson, 1989). TNF induces migration but inhibits proliferation of endothelial cells (Leibovich, Polverini, Shephard, Wiseman, Shively and Nuseir, 1987; Frater-Schroder, Risau, Hallman, Gautschi and Bohlen, 1987), is angiogenic *in vivo* and augments expression of MHC class I on endothelial cells (Poher, Gimbrone, Lappierre, Mendrick, Fiers, Rothlein and Springer, 1987a).

TNFs also stimulate expression of MHC class I and II antigens, leukocyte associated adhesion molecules and polymeric immunoglobulin receptors on secretory epithelial cells. They activate polymorphonuclear (PMN) leukocytes, osteoclasts and bone resorption, and have antiviral activity (Beutler and Cerami, 1986; Paul and Ruddle, 1988). TNFs have many functional similarities to and are synergistic to IL-1 in inducing procoagulant activity, neutrophil adhesion molecules and colony stimulating factor-production in a variety of cells (Poher, Lapierre, Stoplen, Brock, Springer, Fiers, Bevilacqua, Mendrick and Gimbrone 1987b; Bevilacqua *et al*, 1986b).

In addition, TNFs play a critical role i.. normal host resistance to infection and to the growth of malignant tumors, serving as immunostimulants and as mediators of inflammatory response (Beutler and Cerami, 1989; Vilcek and Lee; 1991). Based on their anti-tumor activity, TNF- $\alpha$  and - $\beta$  have been used in cancer therapy (Ruddle, Li, Tang, Gray and McGrath, 1987) but the toxic side effects in most patients have led to dose limitation and an unsuccessful outcome (Lenk, Tannenberger, Muller and Shiga, 1988; Creagan, Kovach, Moertel, Frytak, and Kvols, 1988). On the other hand, overproduction of TNF has been implicated as playing a role in a number of pathological conditions, including, cachexia (Beutler, Milsark and Cerami, 1985b; Oliff, 1988), septic shock following infection with gram-negative bacteria (Tracey, Wei, Monague,

Fong, Hesse, Nguyen, Kuo, Beutler, Cotran, Cerami and Lowry, 1988; Nagai, Saigusa, Shimada, Inagawa, Oshima and Iriki, 1988), and meningococcal septicæmia (Waage, Halstein and Espevik, 1987; Tuomanen, 1993). The development of mechanisms for mediating the synthesis and activities of TNFs will obviously have important potential therapeutic implications.

#### **1.6.4.3 Granulocyte-macrophage and granulocyte colony stimulating factors**

Granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) are produced by monocytes, fibroblasts and endothelial cells and exert effects through receptors found on all mononuclear phagocytes (Burgess and Metcalf, 1980). Specific saturable receptors for G-CSF are found on myelomonocytic cell lines, normal bone marrow cells and macrophage tumor cells. Human G-CSF receptors are found only in neutrophilic granulocytic cells (Burgess and Metcalf, 1980).

While G-CSF stimulates formation of granulocyte colonies (Metcalf and Nicola, 1983; Souza, Boone, Gabrielove, Lai, Zsebo, Murdock, Chazin, Bruszewski, Lu, Chen, Barendt, Platzer, Moore, Martelsmann and Welle, 1986), GM-CSF stimulates formation of pure macrophage, eosinophilic, neutrophilic granulocyte and, mixed granulocyte/macrophage colonies (Tomonaga, Golde and Gasson, 1986). GM-CSF also induces neutrophil migration inhibition factor activity, potentiates neutrophil responses to physiological stimuli and enhances antibody-dependent killing by eosinophils (Gasson, Weisbert, Kaufman, Clark, Hewick, Wong and Golde, 1984; Naccache, Faucher, Borgeat, Gasson and DiPersio, 1988; Chan, Salmon, Nimer, Golde and Gasson, 1986; Silberstein and David, 1987).

Both G- and GM-CSF induce migration and proliferation of endothelial cells and the proliferation of marrow fibroblast precursors, but do not stimulate the proinflammatory/prothrombotic properties or induce functions associated with accessory cell activity (Bussolino, Wang, Defilippi, Turrini, Sanavio,

Edgell, Aglietta, Arese and Montovani, 1989; Dedhar, Gaboury, Galloway and Eaves, 1988).

The range and types of biological activities that have been described for the CSFs suggest diverse clinical utility for these proteins. Extensive clinical trials have been undertaken with both GM-CSF and G-CSF for the treatment of various congenital and disease- or chemotherapy-induced neutropaenias and/or pancytopenias (Sherr, 1990). GM-CSF has received FDA-approval for use in autologous bone marrow transplantation, while G-CSF has received approval for the treatment of chemotherapy-induced neutropaenia.

#### **1.6.4.4 Interleukin 6**

Interleukin 6 (IL-6) is a multifunctional protein produced by lymphoid and non-lymphoid cells as well as by normal and transformed cells. The cells that produce IL-6, include, T cells, B cells, monocytes/macrophages, fibroblasts, endothelial cells, hepatocytes, astrocytes, keratinocytes osteocytes, sertoli cells and various carcinomas and sarcomas including, myelomas, glioblastomas, melanomas plus bladder and renal cell carcinomas (Van Snick, 1990). The production of IL-6 in these various cells is upregulated or downregulated by a variety of signals such as mitogenic or antigenic stimulation, lipopolysaccharide, cytokines and viruses (Van Snick, 1990; Houssiau and Van Snick, 1992; Kishimoto, Akira and Taga, 1992)

The effects of IL-6 on different cells are numerous and varied. The effect on B cells is to stimulate differentiation and antibody secretion (Hirano, Teranishi, Lin and Onoue, 1984; Butler, Falkoff and Fauci, 1984; Hirano, Yasukawa, Harada, Taga, Watanabe, Matsuda, Nakajima, Koyama, Iwamtsu, Tsunasawa, Sakiyama, Matsui, Takahara, Taniguchi and Kishimoto, 1986). IL-6 acts as a T-cell co-stimulant with sub-optimal concentrations of phytohaemagglutinin (PHA) and concanavalin A (Con A) to stimulate IL-2

production and IL-2 receptor expression. IL-6 exhibits growth factor activity for mature thymic or peripheral T cells and reportedly enhances the differentiation of cytotoxic T cells in the presence of IL-2 or IFN $\gamma$  (Lotz, Jiriki, Kabouridis, Tsoukas, Hirano, Kishimoto and Carson, 1988; Tosato and Pike, 1988; Uyttenhove, Coulie, and Van Snick, 1988). IL-6 stimulates production of acute phase proteins by hepatocytes (Bauman, Jahreis, Sauder and Koj, 1984) and has colony-stimulating activity on haematopoietic stem cells (Wong and Clark, 1988; Leary, Ikebuchi, Hirai, Wong, Yang, Clark and Ogawa, 1988); stimulates growth of sarcomas and carcinomas (Van Damme, Bjan Beeumen, Decock, Van Snick, De Ley and Billiau, 1988; Eustace, Han, Gooding, Rowbottom, Richens and Heyderman, 1993). Additional biological activities of IL-6 include: induction of neuronal cell differentiation (Satoh, Nakamura, Taga, Matsuda, Hirano, Kishimoto and Kaziro, 1988; Hama, Miyamoto, Tsukui, Nishio and Hatanaka, 1989), the stimulation of megakaryocytic maturation and platelet production (Ishibashi, Shikama, Kimura, Kawaguchi, Uchida, Yamamoto, Okano, Akiyama, Hirano and Kishimoto, 1993), inhibition of B cell growth (Aderka, Moar, Novick, Engelmann, Kahn, Levo, Wallach, and Revel, 1993) and the induction of fever in an IL-1-dependent mechanism (Coceani, Lees, Mancilla, Belizario and Dinarello, 1993).

The various activities of IL-6 described above suggest that this factor might have a major role in the mediation of the inflammatory and immune responses initiated by infection or injury. Although the exact functions of IL-6 *in vivo* are not known, elevated IL-6 levels have been reported to be elevated in a variety of diseases, including autoimmune diseases, mesangial proliferative glomerulonephritis, psoriasis and malignancies such as plasmacytoma and myeloma (Gastl, Abrams, Nanus, Oosterkamp, Silver, Liu, Chen, Albino, and Bander, 1993).

#### **1.6.4.5 Macrophage inflammatory protein 1**

Macrophage inflammatory protein 1 (MIP-1) consists of two closely related proteins, MIP-1 $\alpha$  and MIP-1 $\beta$ , secreted by T cells, B cells, monocyte/macrophages and mast cells (Wolpe and Cerami; 1989). Both these proteins are chemokines with a combination of shared and divergent functions. A mixture of the two subsets of natural but not recombinant MIP-1 attracts neutrophils. Recombinant MIP-1 $\alpha$  and - $\beta$  attract monocytes and show subset specificity. MIP-1 $\alpha$  attracts B cells, cytotoxic T cells and CD4<sup>+</sup> T cells; MIP-1 $\beta$  is less potent but more selective attracting primarily CD4<sup>+</sup> cells with some preference for the 'naive' phenotype of T cells. Both subsets of MIP-1 suppress haematopoietic stem cell proliferation with MIP-1 $\beta$  being less potent. MIP-1 $\alpha$  chemoattracts and degranulates eosinophils (Rot, Krieger, Brunner, Bischoff, Schall and Dahinden, 1992), induces histamine release from mast cells and basophils, and attracts basophils (Alam, Forsythe, Stafford, Lett-Brown and Grant, 1992), while MIP-1 $\beta$  induces adhesiveness in T cells (Tanaka, Adams, Hubscher, Hirano, Siebenlist and Shaw, 1993).

#### **1.6.4.6 Interferon gamma**

Interferon gamma (IFN $\gamma$ ) is primarily a product of activated T lymphocytes. It exerts its effects on specific saturable receptors found on myelomonocytic, lymphoid, mast, endothelial, fibroblastic, neuronal and melanocytic cells (Trinchieri and Perussia, 1985; Perussia, Mangoni, Engers and Trinchieri, 1980).

In addition to its antiviral activity, IFN $\gamma$  inhibits cell growth in presence of TNF $\beta$  (Trinchieri and Perussia, 1985) and induces MHC class I and II antigen expression in target tissues (Wong, Clark-Lewis, McKimm-Breschkin, Harris and Schrader, 1983; Skoskiewicz, Calvin, Schneeberger and Russell, 1985; Giacomini, Tecce, Gambari, Sacchi, Fisher and Natali, 1988; Gerrard,

Dyer, Zoon, Nedden and Siegel, 1988), primes macrophages for tumor killing and enhances natural killer (NK) cytotoxicity (Trinchieri and Perussia, 1985; Pace, Russell, Torres, Johnson and Gray, 1983). IFN $\gamma$  may play an obligatory role in *de novo* induction of cytolytic activity in lymphocyte activated killer (LAK) and cytotoxic T lymphocyte (CTL) cells by upregulating the p55 component of IL-2 receptor (Giovarelli, Santoni, Jemma, Mussoc, Gillfrida, Cavallo, Landolfo and Forni, 1988).

IFN $\gamma$  augments the expression of MHC class I antigen, induces class II antigens and the invariant chain on endothelial cells (Pober, Collins and Gimbrone, 1983; Collins, Korman, Wake, Boss, Kappes, Fiers, Adult, Gimbrone, Strominger and Pober, 1984). By stimulating MHC class II antigen expression, and the production of IL-1 and IL-6, IFN $\gamma$  induces endothelial cells to act as antigen-presenting cells (Hirschberg, Braathen and Thorsby, 1982) but differs from IL-1 and TNF with regard to the effects on endothelial cells in that it does not induce proinflammatory/prothrombotic or proliferative/migratory activities.

## **1.7 ASTROCYTES AND THEIR POSSIBLE ROLE IN THE PATHOGENESIS OF CENTRAL NERVOUS SYSTEM DISEASE**

The late-stage CNS lesion in human African trypanosomiasis (HAT) is characterised by a progressive infiltration of the meninges and the brain parenchyma by mononuclear inflammatory cells as well as diffuse astrogliosis (Adams *et al*, 1986; Poltera *et al*, 1985; Haller *et al*, 1986 ).

During experimental *T. b. brucei* infections in mice, astrocyte activation, as judged by intensity of staining of GFAP, has been reported to occur prior to the development of inflammatory lesions (Hunter *et al*, 1991). This activation correlates with induction of messenger RNA (mRNA) for the cytokines: TNF, IL-1, IL-4 and IL-2 in the brains of infected mice. Cytokines, especially IL-1 $\alpha$  and TNF- $\alpha$  are important in initiating meningeal inflammatory processes

(Ramilo *et al*, 1990; Saukkonen *et al* , 1990). While macrophages are thought to be the major source of TNF and IL-1, other cells such as activated astrocytes and microglia are capable of producing these cytokines (Fontana *et al*, 1982; Lieberman, Pitha, Shin and Shin, 1989). Astrocytes can be induced to synthesize and secrete IL-1, IL-3, IFN and prostaglandin E (PGE) (Fontana *et al*, 1982; Frei, Bodmer, Schwerdel and Fontana, 1985). Astrocytes can also strongly stimulate antigen-reactive T cells and in some situations are capable of suppressing T-cell activation (Wekerle, Linington, Lassman and Meyerman, 1986). It would appear that astrocytes might have a key role in the neuropathogenesis of late-stage trypanosomiasis.

### **1.7.1 Functions of astrocytes**

There are two types of astrocytes, the protoplasmic and the fibrillary astrocytes. The fibrillary astrocytes are widely distributed throughout the CNS and their numbers increase with age. There is a thick layer of fibrillary astrocytes beneath the pia and ependyma. Between these two surfaces, fibrillary astrocytes are intimately associated with neurones and myelinated fibres, and there is a close association with the microvasculature, the basement membrane of all capillaries being enveloped in the foot processes of astrocytes (reviewed by Adams and Graham, 1988).

The protoplasmic astrocytes are largely confined to the grey matter (cortex and basal ganglia) where they closely invest neurones and their processes, except at synapses. The processes of the protoplasmic astrocytes are short, stout and branch more often than the long, fine processes of the fibrillary astrocytes (reviewed by Adams and Graham, 1988). The Bergmann glia are a variant of astrocytes, whose cell bodies are found adjacent to the Purkinje cells in the granular layer and whose processes extend into the molecular layer of the cerebellum (Sommer, Lagenaur and Schachner, 1981).

Astrocytes are involved in a variety of homeostatic, supportive, phagocytic and trophic functions which are essential for the well-being of neurones (Pentreath, 1989). They form gap junctions with one another and with ependymal cells and oligodendrocytes (Tao-Cheng and Brightman, 1988). A special developmental relationship exists between the perinodal astrocyte-processes and the nodal axon membrane. Optic nerve axons show intense sodium ion ( $\text{Na}^+$ ) channel immunoreactivity at the node of Ranvier, and numerous voltage-sensitive  $\text{Na}^+$  channels in the nearby astrocytic processes (Black and Waxman, 1988; Black, Friedman, Waxman, Elmer and Angelides, 1989). Only a few such channels are present in astrocyte processes forming the glia limitans or surrounding the blood vessels, suggesting a role of astrocytes in neurotransmission. In addition, astrocytes maintain the extracellular concentrations of potassium ions ( $\text{K}^+$ ) and of neurotransmitters such as gamma aminobutyric acid (GABA), in the immediate vicinity of neurones and at the node of Ranvier. The regulation of volume and the homeostasis of  $\text{K}^+$ , is suggested to occur by active uptake and special buffering, in which  $\text{K}^+$  is rapidly shunted by current flow through electrically joined astrocytes forming a glial syncytium (Sontheimer, Minturn, Black, Waxman and Ransom, 1990). According to the 'Modulated Boyle Conway Hypothesis', neural activity triggers the local accumulation of  $\text{K}^+$  and chloride ion ( $\text{Cl}^-$ ) together with water in astrocyte processes, allowing local storage of  $\text{K}^+$  for later return to the neurones (Barres, Chun and Corey, 1988).

Astrocytes extend cytoplasmic foot processes onto the basement membrane of all the brain capillaries. They also provide signals for the formation of tight junctions by the endothelial cells which make the blood-brain barrier (Tao-Cheng and Brightman, 1988). The blood-brain barrier is made complete by the lack of fenestrations and vesicular channels in brain endothelial cells. This barrier prevents the passive flux of molecules as small as monovalent ions (Hertz, McFarlin and Waksman, 1990).

Astrocytes are involved in cell to cell interactions and therefore, express surface receptors for at least 20 neuroligands, including, norepinephrine, 5-hydroxytryptamine (5HT), carbachol, glutamine, histamine, adenine triphosphate (ATP), and bradykinin (Lerea and McCarthy, 1991; Salm and McCarthy, 1992). During inflammation, astrocytes are stimulated by a variety of additional signals, including, cell contacts involving adhesion molecules such as the ICAM-1 and the leukocyte function accessory molecule 1 (LFA-1), cytokines and antigen-antibody complexes with or without complement (Neary, Laskey, Van Breemen, Blicharska, Norenberg and Norenberg, 1991). Cyclic adenosine monophosphate (cAMP) stimulation induces development of astrocyte membrane assemblies and alters astrocytic shape, cytoskeletal organisation and substrate phosphorylation (Tao-Cheng and Brightman, 1988; Goldman, Finkbeiner and Smith, 1991; Tao-Cheng, Bressler and Brightman, 1992).

Astrocytes also perform many of the functions usually attributed to accessory cells of the immune system, such as the release of a variety of cytokines, including, TNF- $\alpha$ , TNF- $\beta$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IFN $\gamma$  and granulocyte-macrophage colony stimulating factor (Benveniste, Sparacio, Norris, Grennet and Fuller, 1990; Chung and Benveniste, 1990; Fontana, Hangartner, de Tribolet and Weber, 1984; Frei, Malipiero, Leist, Zinkernagel, Schwab and Fontana, 1989; Lieberman *et al*, 1989; Malipiero, Frei and Fontana, 1990). In addition, astrocytes are capable of expressing class I and II major histocompatibility complex (MHC) molecules (Dhib-Jalbut and McFarlin, 1989; Wong, Bartlett, Clark-Lewis, Battyre and Schrader, 1984) and stimulate antigen specific T cells in an MHC-restricted manner (Fierz, Endler, Reske, Wekerle and Fontana, 1985; Dhib-Jalbut, Kufta, Flerlage, Shimijo and McFarlin, 1990). Astrocytes can be stimulated to produce cytokines with diverse factors such as cytokines, lipopolysaccharide and neurotropic viruses

(Benveniste *et al*, 1990; Chung and Benveniste, 1990; Fontan *et al*, 1984; Frei *et al*, 1989; Lieberman *et al*, 1989; Malipiero *et al*, 1990)

Astrocytes may be the primary facultative antigen-presenting cells regulating immune reactivity within the CNS parenchyma. Their ubiquitous distribution and special arrangement at the blood-brain interface are suitably designed for collecting and concentrating antigenic determinants around the blood vessels, therefore protecting the parenchyma by focusing the immune reactions and allowing rapid clearance of toxic metabolites in the blood. The capacity of astrocytes to produce immunosuppressive mediators such as PGE, appears additionally important in potentially limiting reactions away from this immune privileged site. In late-stage sleeping sickness, activated astrocytes may interact locally with activated T-cells and produce cytokines, which may in turn produce concomitant B-cell response and antibody secretion (Pentreath *et al*, 1990)

### **1.7.2 Astrocytes in central nervous system disease**

Following many types of insults to the CNS, astrocytes increase in number, become large and extend more processes and significantly increase their cytoplasmic content of glial fibrillary acidic protein (GFAP), an astrocyte-specific intermediate filament. These cells are called reactive astrocytes and the response is referred to as reactive gliosis (Bignami, Eng, Dahl and Uyeda, 1972; Bignami and Dahl, 1976; Latov, Nilayer, Zimmerman, Johnson, Silverman, Dejandini and Cote, 1979; O'Callaghan, Miller and Reinhard, 1990; Smith, Gibbs, Forno and Eng, 1987). Reactive gliosis is initiated within hours of injury and over time the process results in the formation of a 'gliotic scar', which may be beneficial when it walls off abscesses or detrimental when it interferes with nervous remyelination as in Multiple Sclerosis (MS) (Adams, Graham and Gennarelli, 1983). Signals that induce astrocyte reactivity after injury remain incompletely defined. If such signals could be identified at least

for specified injuries and means were available to inhibit their activity, then the detrimental end-stage glial scars and reactive encephalopathies might be avoided.

Specific cytokines, including, IL-1 (Giulian and Lachman, 1985), TNF- $\alpha$  and IL-6 (Selmaj, Farooq, Norton, Raine and Brosnan, 1990) have been reported to stimulate neonatal rodent and bovine astrocytes in culture. In addition, TNF has been shown to promote proliferation of adult human astrocytes *in vitro* (Barna, Estes, Jacobs, Hudson and Ransohoff, 1990). Certain cytokines released by inflammatory mononuclear cells (MNC) can provide the cellular signals to initiate the process of reactive gliosis in the pathological brain conditions where the MNC are prominently featured. For instance, the lymphokine IFN- $\gamma$ , induces proliferation of human adult astrocytes *in vitro* and increases the extent of trauma-induced gliosis in adult mouse brain (Yong, Moumdjian, Yong, Ruijs, Freedman, Cashman and Antel, 1991).

In addition to astrocytes, MNCs, namely, the lymphocytes and blood-derived macrophages and the intrinsic microglia cells are prominently featured in many pathological conditions of the brain, such as, multiple sclerosis, traumatic injuries (Giulian, Chen, Ingeman, George and Naponnen, 1989), viral infections and parasitic diseases including African trypanosomiasis (Adams *et al*, 1986; Poltera, 1985; Hunter *et al*, 1991). These cells interact with each other and other cells, such as the astrocytes, via soluble mediators, including, adhesion molecules, antibodies and cytokines, to propagate the CNS inflammatory process. It is highly likely that astrocytes play a major role in the pathogenesis of the CNS lesions that occur during late-stage trypanosomiasis.

## 1.8 THE MOUSE MODEL

The mechanisms of pathogenicity of the CNS trypanosomiasis and the adverse reactions resulting from some drug treatments are poorly understood and remain a considerable challenge to scientists. Early studies (Mott, 1906) described meningoencephalitis in cases of sleeping sickness. Later the neuropathology of untreated (Van Bogeart, 1958), and insufficiently treated patients (Calwell, 1937), as well as myocarditis (Hawking and Greenfield, 1941), were documented. Once trypanosomiasis was brought to an acceptable level of control, autopsy cases became rare (Cohen, 1973; Poltera *et al*, 1977) and this situation still exists. This highlights the need for animal models that closely mimic human trypanosomiasis to facilitate studies on the pathogenesis and pathology of human African trypanosomiasis, and the adverse drug reactions associated with trypanocidal therapy.

Jennings *et al* (1977) used two diminazene aceturate- (Berenil<sup>R</sup>, Hoechst) susceptible stabilates of *T.b. brucei* to investigate the source of relapsing parasitaemia in mice. They found that relapses consistently occurred if treatment was delayed by 21 or more days following infection, and that these relapses were not dependent on dosages of the drug used or the size of the parasite inoculum. Similar relapses occurred when other trypanocides (samorin, ethidium and prothidium), that do not cross the blood-brain barrier, were used instead of Berenil. It was later demonstrated that the brain was the source of the relapse infections in such mice (Jennings, Whitelaw, Holmes, Chizyuak and Urquhart, 1979; Jennings and Gray, 1983) and that these relapses resulted from insufficient treatment of the late-stage disease since parasites isolated from relapse infections were not drug resistant. This mouse system reflects all the main stages of human sleeping sickness and is now being widely utilised as a model to study the disease and evaluate the efficacy of chemotherapeutic agents for trypanosomiasis.

Although *T.b.brucei* is not infective to man, it belongs to the same subgenus (*Trypanozoon*) as the two human infective parasite species (Hoare, 1970). The similarities include tissue invasiveness, and causing chronic infection in domestic and laboratory animals with similar characteristics to the human disease, such as, the involvement of the CNS (Goodwin, 1974; Poltera *et al*, 1980a; Jennings *et al*, 1989).

### **1.8.1 The clinical syndromes**

This mouse model presents three clinical syndromes: the early-stage, the late-stage and the advanced stage.

#### **1.8.1.1 Early-stage**

This is the stage in the *T.b. brucei*-infections of the mouse model that is sensitive to all conventional trypanocides including, those that do not cross the blood-brain barrier to any significant extent. For the mouse model used in this study, this coincides with the duration between day 1 and 21 of infection. This is the stage of infection in the mouse model that is equivalent to early-stage human African trypanosomiasis.

This stage is characterised by reduced activity, raised hair coats, hunched posture, enlarged abdomen due to splenomegaly, and by high parasitaemia. The parasitaemia is detectable from day 3 or 4 reaching a peak on day 7 to 9 after infection.

Mice in the early stage of infection show no histopathological changes in the brain although some parasites can be detected in the choroid plexus from day 14 onwards.

#### **1.8.1.2 Late-stage**

Mice in this stage of infection are not cured by treatment with early-

stage trypanocides such as Berenil, samorin, ethidium and prothidium (Jennings *et al*, 1977; Jennings and Gray, 1983). In the mouse model, this stage consists of two groups of animals: mice with infections of more than 21 days duration but which have not been given any trypanocidal therapy, and mice that receive subcurative Berenil therapy on or after day 21 of infection. In mice that have had no trypanocidal therapy, this stage coincides with the duration from day 21 after infection to the end of the disease course. If mice are treated with Berenil on or after day 21, then this stage lasts for the duration between this treatment and the relapse of parasitaemia, which occurs 20 to 22 days later.

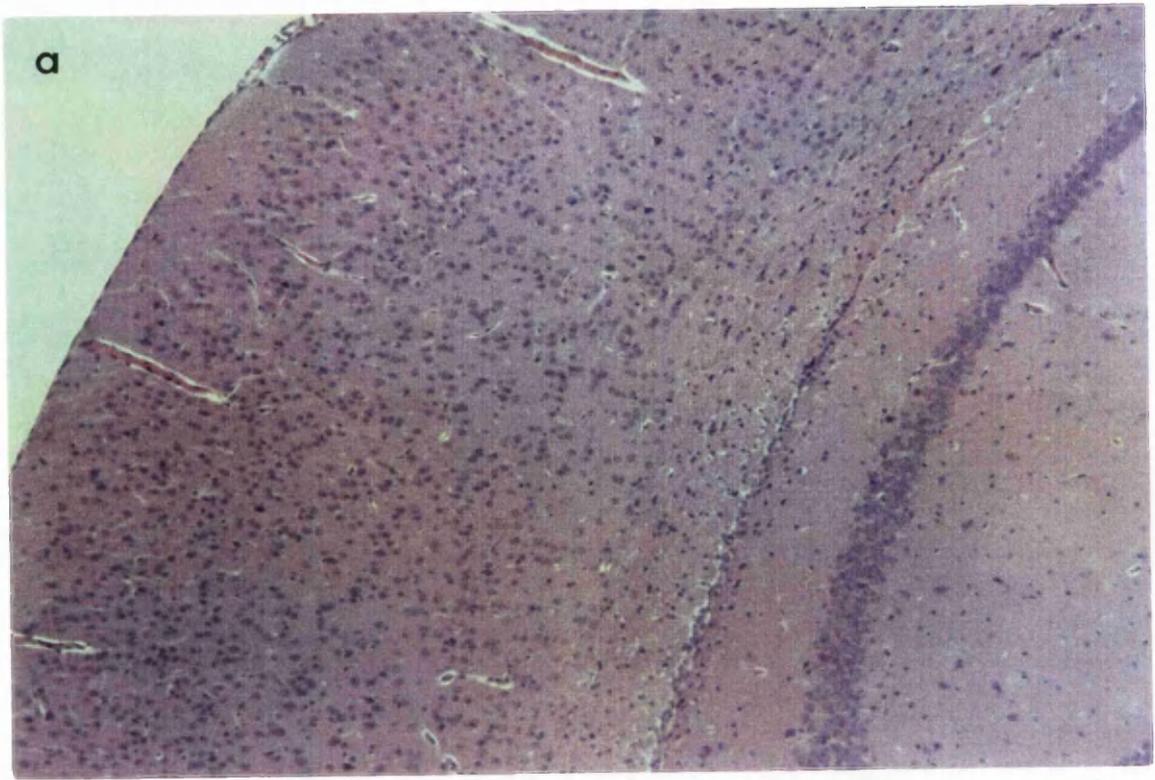
The late-stage infection is characterised by waves of parasitaemia, each wave with a lower peak than the previous wave (Jennings *et al*, 1977; Anosa, Jennings and Urquhart, 1977). The mice are dull, progressively lose weight and show intermittent episodes of raised hair coats and hunched postures, corresponding to the peaks of parasitaemic waves (personal observation). By day 43 after infection onwards, mice show slight tremors, and progressively lose the ability to hang upside down from the roof of the cage. Occasionally, ataxia and hind limb paralysis occurs (F.W. Jennings personal communication and personal observations). These signs reflect CNS involvement. This stage is equivalent to the late-stage human African trypanosomiasis.

The two groups of mice, in the late-stage of infection, show similar pathological changes but the mice that receive subcurative Berenil treatment develop the changes faster than the untreated mice. Figure 1.1 was included to provide a comparison of the diseased state to the normal mouse. In the untreated mice, mononuclear cellular infiltration into the subarachnoid and perivascular spaces, and the choroid fissure, occur from day 28 of infections onwards (Figures 1.2 and 1.3). The infiltrating cells consist of lymphocytes, plasma cells and macrophages. This infiltration starts as a single layer of infiltrating cells but as the disease progresses, the cellular infiltrations increase in size, and Russell-body containing plasma cells can be seen in these infiltrations (Jennings *et al*,

**Figure 1.1**

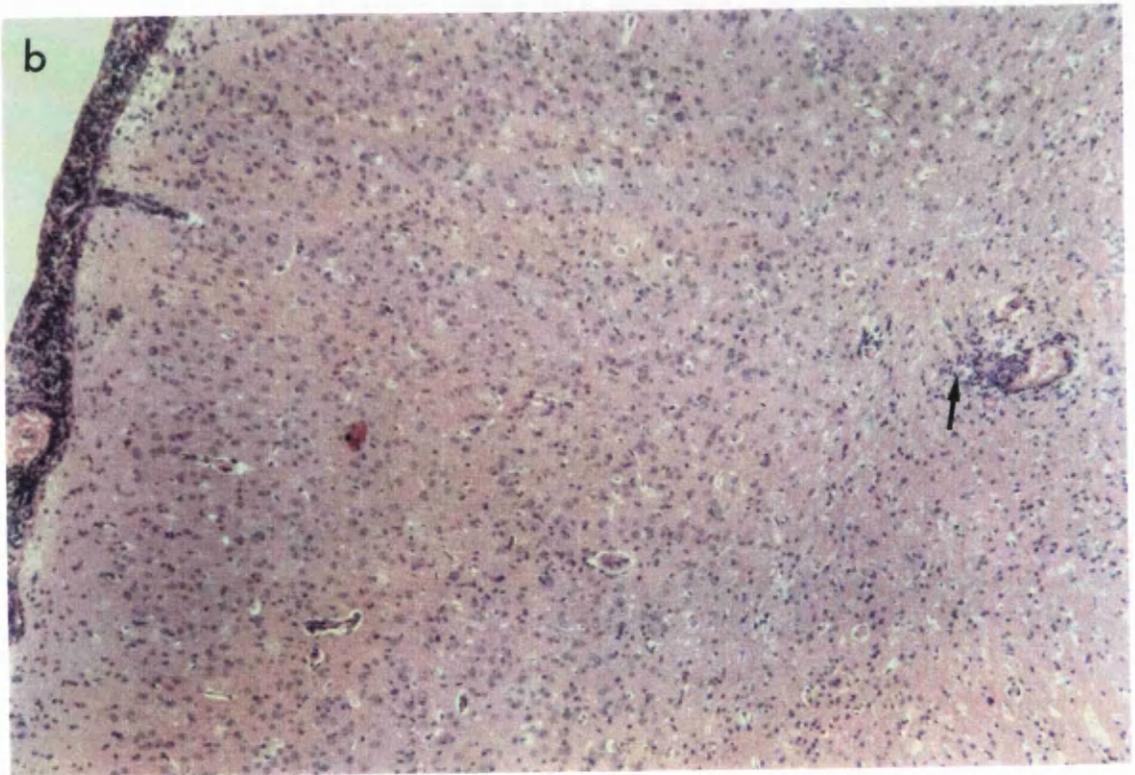
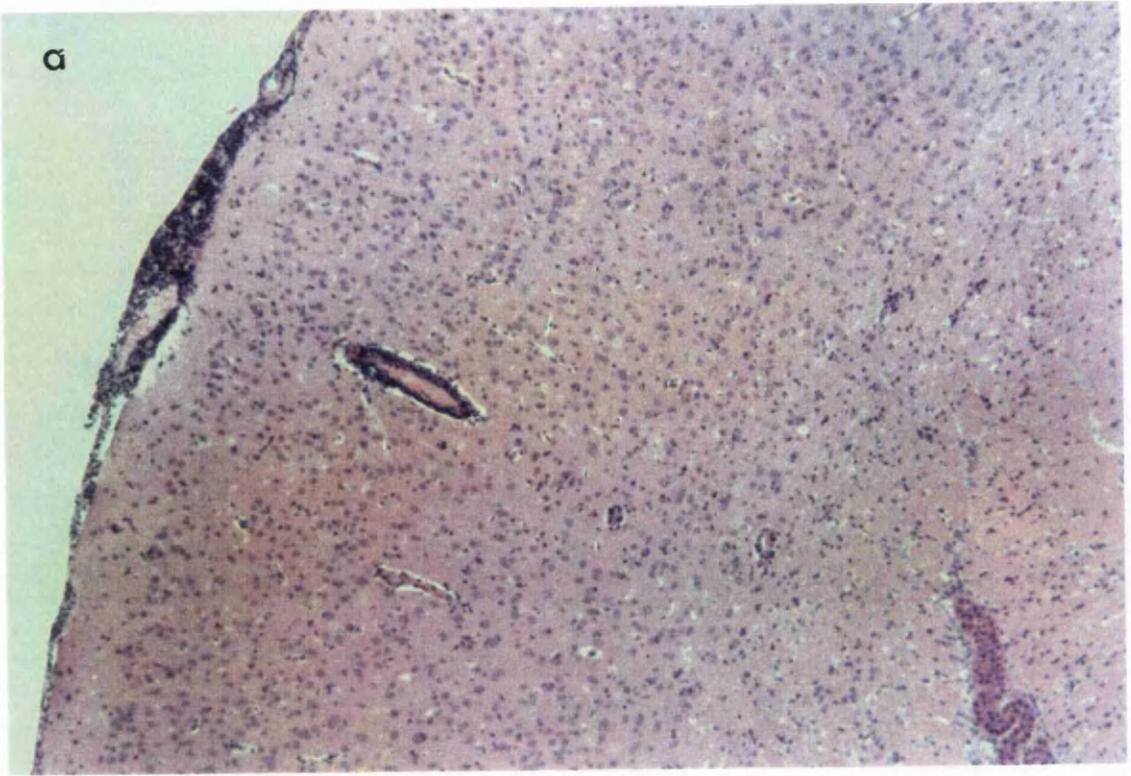
The cerebral cortex (a) and the cerebellar cortex (b) of a normal mouse.

Haematoxylin and eosin. x100



**Figure 1.2**

Cerebral sections from mice killed in the late (a) and the advanced (b) stages of infection with *T. b. brucei* GVR 35, showing infiltration of the meninges and the perivascular spaces by inflammatory cells. 1.2b shows the increment in the cellular infiltrates into the meninges and a burst perivascular cuff (arrow) that is spilling inflammatory cells into the adjacent parenchyma. Haematoxylin and eosin. x100



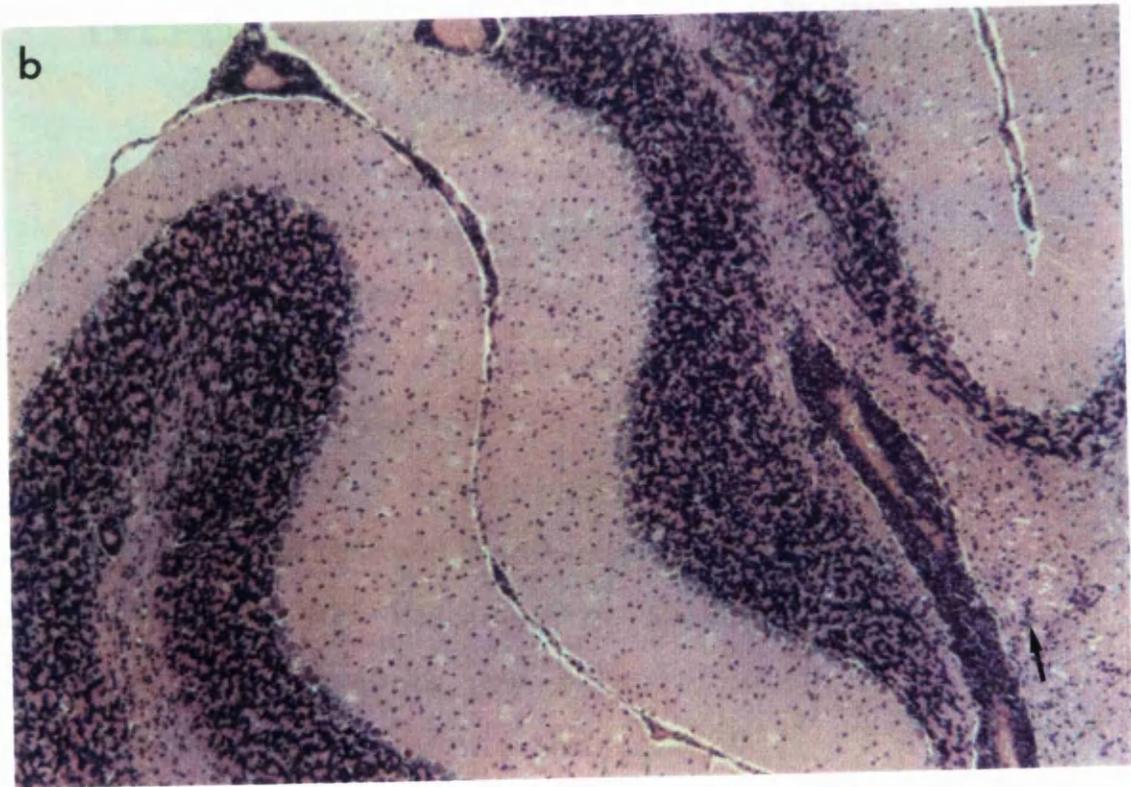
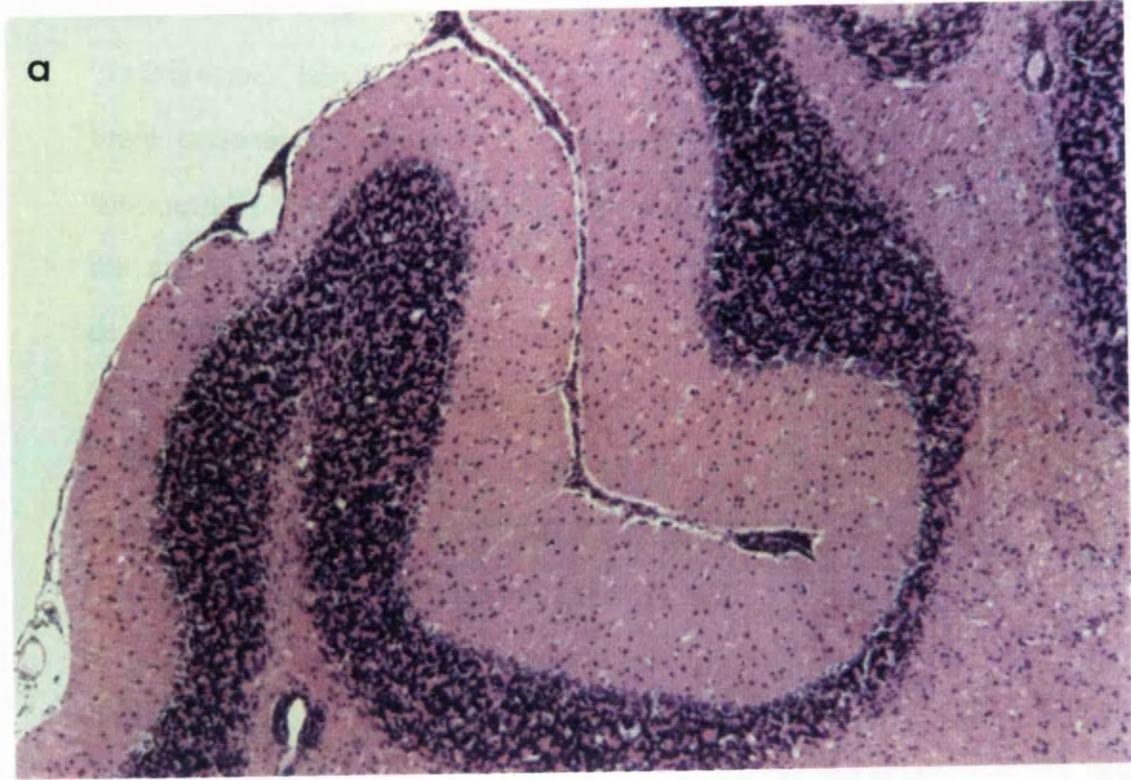
### Figure 1.3

The hippocampal area of a mouse killed in the advanced stage of *T.b. brucei* GVR 35 infection. It features numerous inflammatory cells infiltrating the choroid fissure and perivascular cuffs within the hippocampus, some of which are burst and spilling cells into the adjacent parenchyma (arrows). Haematoxylin and eosin. x100



#### **Figure 1.4**

Cerebellar cortices of mice killed in late (a) and advanced (b) stages of infection with *T.b. brucei* GVR 35, showing infiltration of the meninges, along the cerebellar lobes, by inflammatory cells and perivascular cuffs. Note that in the advanced stage (b) inflammatory cells can be seen in the white matter adjacent to the large perivascular cuff (arrow). The sections were stained with haematoxylin and eosin. Magnification. x100



1989; Hunter *et al*, 1991; personal observations); see Figures 1.2a and 1.4a. Trypanosomes can be seen mainly in the choroid plexus and occasionally in the brain parenchyma (Anosa *et al*, 1977; personal observations). In mice treated subcuratively with early-stage drugs, the disease progresses faster. Thus, while the pathological lesions shown in Figures 1.2a, and 1.4a, took 72 days to develop in untreated mice, it took only 22 days after the subcurative therapy (total of 43 days) for similar changes to develop (personal observation). Following subcurative therapy, trypanosomes are found only in the brain parenchyma until relapse of parasitaemia when the choroid plexus is re-invaded (Jennings and Gray, 1983; personal observations). Neuronal degeneration can be seen in the late-stage of infection, particularly in mice that receive Berenil therapy (personal observation). The demarcation between the pathology in the late and advanced stages of infection is not clear cut. Sometimes the changes in this stage are as severe as those found in the advanced stage of infection.

### **1.8.1.3 Advanced-stage**

Treatment of mice infected with *T.b. brucei* GVR 35 after 21 days of infection with early-stage trypanocides, such as Berenil, clears the parasites in the systemic circulation and in tissues other than the CNS. The parasites that remain within the brain parenchyma continue to replicate and give rise to recrudescence parasitaemia (Jennings *et al*, 1977; Jennings *et al*, 1979; Jennings and Gray, 1983). Non-curative treatment of relapsed infections results in exacerbation of the existing meningoencephalitis creating changes similar to those seen in the brains of human reactive arsenical encephalopathy victims (Adams *et al*, 1986; Haller *et al*, 1986; Jennings *et al*, 1989; Hunter *et al*, 1991). This is the advanced stage of infection in the mouse model and is taken to represent the human reactive arsenical encephalopathy (RAE). Since the drug used to simulate RAE, in the studies carried out in the mouse here, is not arsenical, the reaction created is referred to as post-treatment reactive

encephalitis (PTRE). Mice in this stage of infection are dull, ataxic and emaciated. There is a high incidence of hind limb paralysis (personal observations). Some mice may still have enlarged spleens reflected as enlarged abdomens.

Mice that receive a second Berenil treatment after relapse of parasitaemia, show exacerbations of the CNS histopathological changes (Jennings *et al*, 1989; personal observations). The infiltrations in the subarachnoid and the perivascular spaces enlarge; some of the perivascular cuffs rupture, releasing inflammatory cells into the adjacent parenchyma (Fig 1.2b, 1.3 and 1.4b). In addition, inflammatory cells and trypanosomes, not associated with blood vessels, as well as neuronal degeneration, can be seen in the parenchyma (Figure 1.5). The fore-, mid- and hindbrain are affected equally.

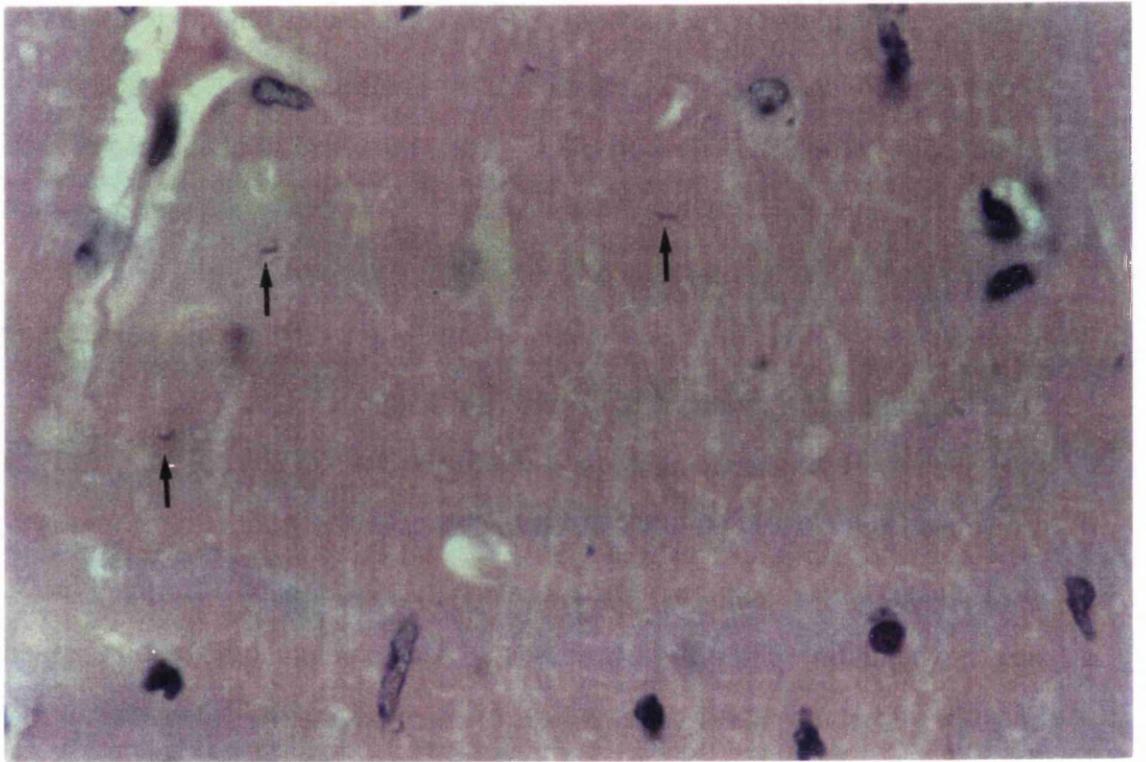
In summary, the pathology in the CNS of the mouse model, is a slowly progressive infiltration of the meninges, the perivascular spaces and eventually the brain parenchyma by cells of the lymphocyte/plasma series and macrophages, and occasional neuronal degeneration. The development of these changes can be hastened by subcurative therapy with trypanocidal drugs, including Berenil, on or after 21 days of infection.

## **1.9 JUSTIFICATION AND AIMS OF THIS STUDY**

Despite the fact that HAT has been recognised clinically since the turn of the century, relatively little is known about the pathogenesis of the disease. The event that ultimately leads to the development of CNS disease is the invasion of the brain by trypanosomes (Greenwood and Whittle, 1973; 1980; Schultzberg *et al*, 1988). Reports on the clinical signs and the pathological changes in the brains of HAT patients during trypanosome infections and the adverse reactions that occur after treatment with melarsoprol are well documented (Greenwood

**Figure 1.5**

Hippocampal parenchyma of a mouse in advanced stage of infection with *T.b. brucei* GVR 35, showing trypanosomes scattered in the parenchyma, not associated with blood vessels (arrows). Haematoxylin and eosin. x1000



and Whittle, 1980; de Raadt, 1984; Adams *et al*, 1986; Haller *et al*, 1986, Cegielski and Durack, 1991). According to these accounts the major histopathological changes are infiltration of the subarachnoid and Virchow Robin spaces by cells of the lymphocytes/plasma cell series and macrophages, accompanied by diffuse gliosis. Neuronal degeneration is reported to occur only in the very advanced stages of the disease (Adams *et al*, 1986).

Various hypotheses regarding the genesis of CNS disease during HAT and the adverse drug reaction after melarsoprol therapy have been proposed (reviewed by Hunter and Kennedy, 1992; section 1.6). These include: changes brought about by trypanosome-produced toxins, such as phospholipases which lead to production of cytokines and PGD<sub>2</sub> production, leading to inflammatory changes and slow wave sleep (Pentreath *et al*, 1990); induction of harmful metabolic changes such as the production of indole-ethanol and phenylpyruvate from aromatic amino acids, which may lead to depressed levels of neurotransmitters and inhibition of mitochondrial function, respectively (Seed *et al*, 1983; Patel *et al*, 1977); and the host's immune response to the invading parasite or parasite fractions (Greenwood and Whittle, 1980; Lambert *et al*, 1981; Pepin and Milord, 1991). Thus, the CNS pathology could result from: the tissue disruption that is caused by edema and infiltrating inflammatory cells and trypanosomes (Adams *et al*, 1986; Haller *et al*, 1986); the local production of immunoglobulins and immune complexes that activate the complement and Kallekrein-kinin systems (Lambert *et al*, 1981; Boreham, 1985); the autoantibody production against brain cells or against trypanosome antigens adhering on the brain cells (Asonganyi *et al*, 1979; Rifkin and Landsberger, 1990); and mediators and lytic enzymes produced by the infiltrating cells (Boreham, 1985; Pentreath *et al*, 1990; Hunter *et al*, 1991). Experimental infections of mice with *T.b.brucei*, followed by curative and subcurative treatment of these infections, seem to support the view that the CNS pathology is a consequence of the host's immune reaction to trypanosomes within the brain

parenchyma (Hunter *et al*, 1992c).

Encephalopathy is a common and catastrophic complication of the treatment of late stages of HAT with melarsoprol (de Raadt, 1984; Poltera, 1985; Veeken, Ebelings and Dolmans, 1989), but its pathogenesis remains obscure. Jennings *et al* (1989) suggested that it is caused by ineffective subcurative therapy that leads to rapid disappearance of parasites from the blood stream with persistence of trypanosomes in the CNS, concentrating the immune reaction to the CNS. While melarsoprol, a late-stage drug, induces encephalopathy (de Raadt, 1984; Adams *et al*, 1986; Haller *et al*, 1986; Pepin *et al*, 1989), clinical experience with early-stage drugs in late-stage human patients yielded equivocal results (de Raadt, 1966; Pepin and Milord, 1991). Arsenical toxicity has been suspected as a cause of encephalopathy but it has been shown that use of the heavy metal chelator, dimercaprol, does not prevent its fatal outcome and that patients who recovered from episodes of encephalopathy could receive further doses of melarsoprol (Pepin and Milord, 1991). Efficacy of prednisolone in preventing arsenical encephalopathy (Pepin and Milord, 1991) implicates an immune mechanism. Another suggested cause of encephalopathy is the deposition of immune complexes in the brain but, while the levels of immune complexes in the blood rise during trypanosomal infections, only minimal amounts of immune complexes have been found in cerebrospinal fluid of late-stage patients (Whittle *et al*, 1980; Lambert *et al*, 1981) suggesting that immune complexes are not a major cause. Autoimmunity due to cross-reacting antigens or due to trypanosome antigens attaching onto the neuronal cell surfaces is not supported by the presence of mononuclear cells in the lesion (Haller *et al*, 1986), instead of polymorphonuclear cellular infiltrates which would normally associated with autoimmune reactions.

Precisely how the trypanosomes invade the CNS, how the inflammatory cells infiltrate the invaded areas, and how the cellular infiltrations and the

diffuse gliosis, that follow trypanosome invasion of the CNS, might affect the cerebral functions are unanswered questions. Recent studies have focused on the role of astrocytes in HAT (Pentreath *et al*, 1989). The facts that astrocytosis occurs in HAT (Adams *et al*, 1986; Haller *et al*, 1986) and in experimental *T.b. brucei* infections in mice (Hunter *et al*, 1991), that astrocytes produce cytokines, such as IL-1 and TNF, that can mediate meningeal inflammation (Fontana *et al*, 1982; Lieberman *et al*, 1989; Hertz *et al*, 1990) and that astrocyte activation correlates with production of such cytokines in the brains of *T.b. brucei*-infected mice (Hunter *et al*, 1991), strongly implicate astrocytes as initiators of the CNS pathology. An understanding of how astrocytes respond to trypanosomes and how these responses can be prevented or attenuated might help to provide better methods of management of HAT.

The aims of this study are:

1. To investigate when astrocyte activation occurs in the brain of *T.b. brucei*-infected mice, how this activation relates to the inflammatory cell infiltration and how it is affected by curative and sub-curative trypanocidal therapy
2. To investigate the cytokines produced by cultured astrocytes after *in vitro* exposure to trypanosomes and trypanosome fractions including, the whole trypanosome lysate and purified VSG.
3. To investigate how eflornithine and azathioprine, two drugs reported to prevent and attenuate inflammatory cell infiltrations in the brains of *T.b. brucei*-infected mice, affect astrocyte activation *in vivo* and *in vitro*.

## **CHAPTER 2**

### **GENERAL MATERIALS AND METHODS**

## **2.1 ANIMALS**

### **2.1.1 Adult mice**

Adult female NIH mice were purchased from Charles River Limited, England. They were allowed to acclimatise to the animal house facilities at the Veterinary School, Garscube for at least 3 weeks before being infected with trypanosomes. Feed and water were provided *ad libitum* throughout their stay in the animal house facilities.

### **2.1.2 Neonate mice**

Ten NIH breeding pairs of mice were purchased from the Harlan Olac farm, England, and were used to establish a colony in the animal house facilities at the Southern General Hospital, Glasgow. Two to 3-day-old litters from these mice were used for all the *in vitro* astrocyte cultures used for this study.

## **2.2 TRYPANOSOMES**

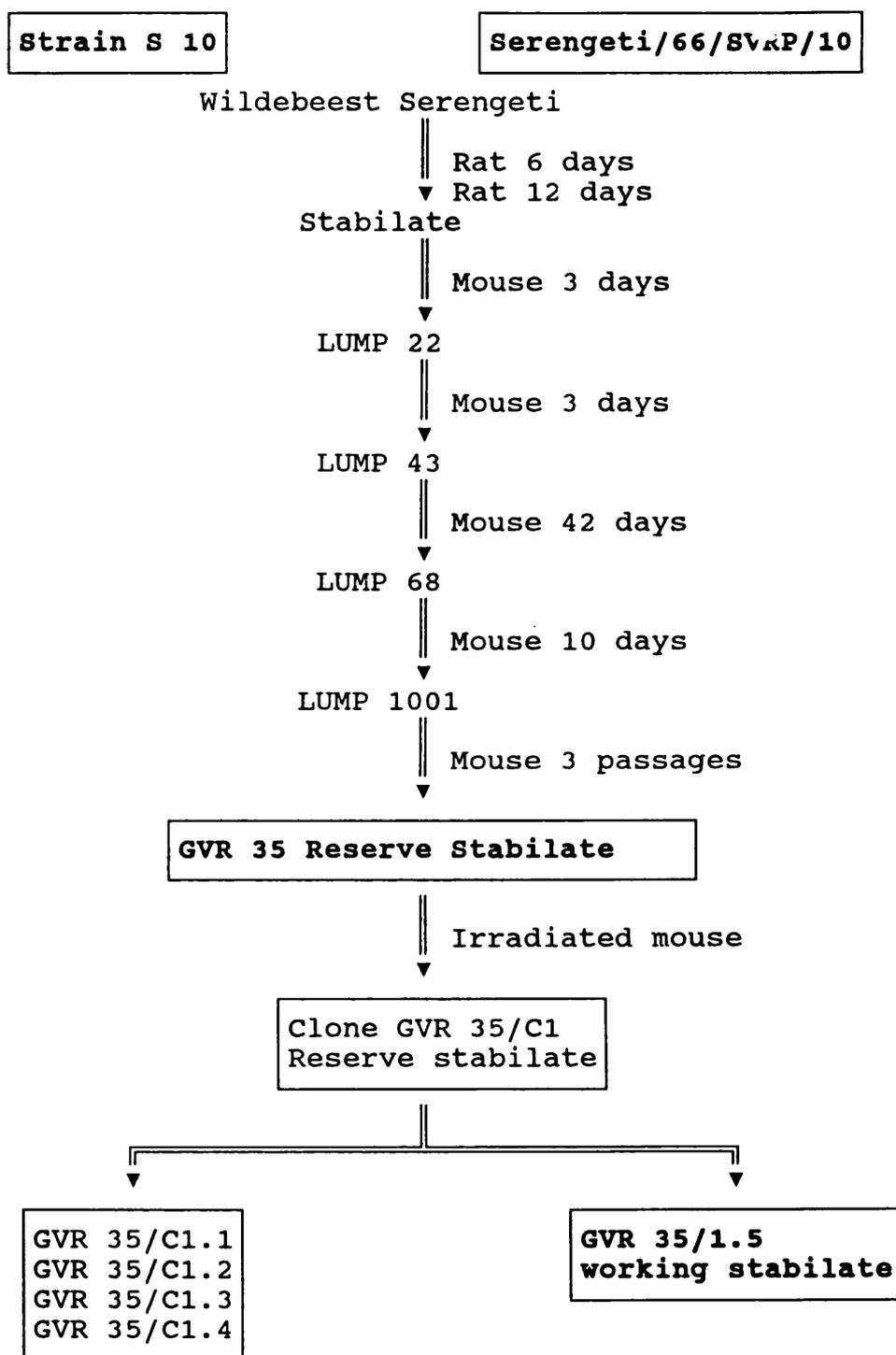
All the trypanosome stabilates used in this study were a generous gift from Dr. F.W. Jennings of the Department of Veterinary Parasitology, University of Glasgow.

### **2.2.1 Eflornithine-sensitive trypanosomes**

The eflornithine-sensitive cloned stabilate was derived from trypanosomes originally isolated from a wildebeest in the Serengeti National Park in 1966 (Serengeti/66/SVRP/10). It was obtained from London School of Hygiene and Tropical Medicine as the stabilate *T. brucei* LUMP 1001 in 1977. A clone was grown from this and a reserve stabilate, GVR/35/C1 was established and maintained in liquid nitrogen (Jennings *et al*, 1977). As required new working stabilates were prepared from this reserve and in the current studies *T.b. brucei* stabilate GVR/35/C1.5, was used (Table 2.1).

Table 2.1

The history of the eflornithine-sensitive *T.b.brucei* stabilate GVR 35/C1.5



Sub-lethally irradiated mice were inoculated i.p. with approximately  $4 \times 10^4$  trypanosomes prepared directly from the frozen stabilate in phosphate-buffered glucose saline (PBGS), pH 8.0. When the parasitaemia had reached antilog 8.1 (Herbert and Lumsden, 1976), the mouse was bled via a cardiac puncture, under terminal anaesthesia. This blood was subsequently used to infect experimental mice, each mouse receiving 0.2ml of the blood/PBGS mixture containing approximately  $4 \times 10^4$  trypanosomes i.p.. Fresh blood from tail snips was examined for parasites twice every week throughout the course of the experiments. At least 20 fields at 400x magnification were examined before a sample was designated negative.

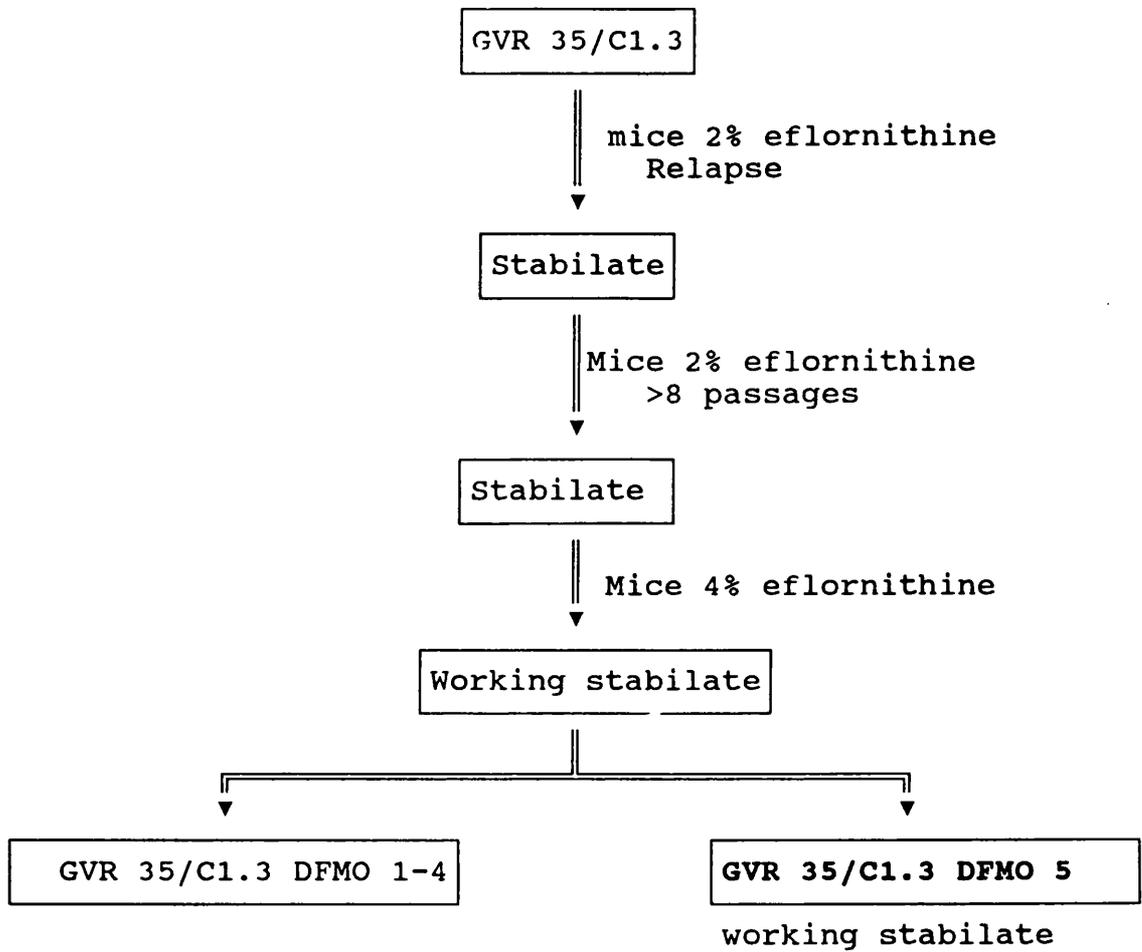
### **2.2.2 Eflornithine-resistant trypanosomes**

Mice were infected with  $4 \times 10^4$  *T.b. brucei* GVR/35/C1.3 which was prepared from the same reserve stabilate as GVR/35/C1.5. Twenty one days after infection 2% eflornithine was administered for a period of 15 days in drinking water. The mice were maintained in a 2 hours light and 4 hours dark environment to ensure an evenly distributed intake of eflornithine (Gillet, Bone, Lowa, Rona and Schechter, 1986), throughout the duration of study.

At the end of 15 day period, eflornithine administration was discontinued and the mice were monitored for relapsed parasitaemia. When this occurred, a single relapsed mouse was bled and the infected blood was used to infect a new batch of 6 normal mice which were then treated with eflornithine, as before, at 21 days. This process was repeated more than 8 times taking a duration of over one year, by which time the resultant infection was completely refractory to treatment with 4% eflornithine, which would, otherwise, have been curative. The trypanosomes were stabilated as *T.b. brucei* GVR/35/1.3 DFMO 5 (Table 2.2).

**Table 2.2**

**The history of the eflornithine-resistant *T.b.brucei* stabilate GVR 35/C1.3 DFMO 5**



## **2.3 INFECTION AND TREATMENT REGIMENS**

### **2.3.1 Infection without treatment**

A group of adult female NIH mice were infected with  $4 \times 10^4$  parasites of either the eflornithine sensitive stabilate *T.b. brucei* GVR 35/C1.5 or the eflornithine resistant stabilate GVR 35/C1.3 DFMO 5. At this inoculation dose, these stabilates have been reported to produce parasitaemia in mice within 3 days of infection. The infection was allowed to proceed without any trypanocidal therapy to the terminal stages of infection. During the course of the disease, mice died as a result of infection, with few surviving beyond day 52 of infection. Five mice were sacrificed every 7 days

### **2.3.2 Induction of post-treatment reactive encephalitis**

Adult female NIH mice were infected with  $4 \times 10^4$  trypanosomes of either the eflornithine-sensitive stabilate *T.b. brucei* GVR 35/C1.5 or the eflornithine-resistant stabilate *T.b. brucei* GVR 35/C1.3 DFMO 5. They were then divided into two groups. One group, P1, was treated with 40mg/kg diminazene aceturate (Berenil, Hoechst) on day 21 after infection. Five mice were killed every 7 days thereafter. At this dosage, Berenil has been reported to give maximal trypanocidal effect without toxicity to mice (Jennings *et al*, 1977). Treatment of *T.b. brucei*-infected mice with drugs that do not cross the blood-brain barrier such as Berenil, after 21 days of infection, induces a PTRE (Jennings *et al*, 1977; Jennings *et al*, 1989; Hunter, Jennings, Adams, Murray and Kennedy, 1992a). The second group, P2, received 40mg/kg Berenil on day 21 of infection and again after a relapse of parasitaemia. This relapse occurred 20-22 days after administration of the first Berenil. This second Berenil treatment has been reported to induce a very severe PTRE (Jennings *et al*, 1989; Hunter *et al*, 1992a). Five mice were sacrificed every 7 days after the second Berenil treatment.

### **2.3.3 Evaluation of curative trypanocidal therapy on astrocyte activation**

A combination of the arsenical drug, Mel Cy (Cymelarsen, Rhone Merieux, France) and a nitroimidazole compound, MK436 (Merck Institute for Therapeutic Research, New Jersey, U.S.A.), has been reported to cure chronic *T.b. brucei* infections in the mouse model (Jennings, 1991c). This combination was, therefore, used in the current study for the evaluation of astrocyte activation after curative therapy.

Mice were infected with either the eflornithine-sensitive or eflornithine-resistant *T.b. brucei* stabilates and treated with 40mg/kg Berenil on day 21 after infection and again after relapse of parasitaemia. Seven days after the second Berenil, a group of these mice, CT, were treated with two consecutive daily doses of 5mg/kg Mel Cy + 15mg/kg Mk436 i.p. Groups of five mice were serially sacrificed 5 days after the combined Mel Cy/MK436 therapy and every 7 days thereafter.

### **2.3.4 Evaluation of the effect of eflornithine and azathioprine on the induction of the post-treatment reactive encephalitis**

#### **2.3.4.1 Eflornithine**

Adult female NIH mice were infected with the eflornithine resistant *T.b. brucei* stabilate, GVR 35/C1.3 DFMO 5. They were then divided into 4 groups (Table 2.3). Two percent eflornithine was administered *ad libitum* in drinking water for a duration of 7 or 14 days, in a 2-hour-light-4-hour-dark environment to spread the drug intake evenly (Gillet *et al*, 1986). Eflornithine administration was arranged so that the treatment with Berenil was at the beginning or end of eflornithine course, or on the 7<sup>th</sup> day of eflornithine therapy. Thus, one group, E1, received eflornithine from the time of Berenil treatment on day 21 after infection. Two other groups, E2 and E3 received eflornithine for 7 and 14 days, respectively, before the treatment with Berenil on day 28 after infection.

**Table 2.3**  
**Treatment schedules for evaluation of the effect of eflornithine and azathioprine on the induction of post-treatment encephalitis**

E1	I	0	<pre> EEEEEEEEEEEE   B-----K-----K-----K-----K-----K   21 28 35 42 49 56 EEEEEE   B-----B-----B-----B-----B-----B   14 21 28 35 42 49 56 </pre>			
E2	I	0	<pre> EEEEEEEEEEEE   B-----B-----B-----B-----B-----B   14 21 28 35 42 49 56 </pre>			
E3	I	0	<pre> EEEEEEEEEEEE   B-----B-----B-----B-----B-----B   14 21 28 35 42 49 56 </pre>			
E4	I	0	<pre> EEEEEEEEEEEEEEEEEEEE   B-----B-----B-----B-----B-----B   14 21 28 35 42 49 56 </pre>			
A1	I	0	<pre> AAAAAAAAA   B-----B-----B-----B-----B-----B   14 21 24 26 32 </pre>			

E1 represents the group of mice that received eflornithine (E) from the day of PTRE induction, i.e., the day of the first Berenil treatment. Groups E2 and E3 were pre-treated with eflornithine for 7 and 14 days, respectively, before the induction of PTRE. E4 represents the group of mice that received eflornithine for 7 days before being treated with Berenil. Eflornithine therapy was continued for further 7 or 14 days. A1 represents the group of mice that received azathioprine for 9 days starting from 2 days before the induction of PTRE. I = infection, B = Berenil treatment, E = eflornithine treatment, A = azathioprine treatment, K = killing time point. Numbers represent time in days after infection.

A fourth group (E4) was treated with eflornithine for 7 days before induction of PTRE with Berenil on day 28 of infection. In this particular group, eflornithine therapy was continued for a further 7 or 14 days, after the Berenil treatment.

Mice, in groups E1 to E4, were sacrificed in groups of 5 at the end of eflornithine course and every 7 days, thereafter. However, the mice that received Berenil at the end of the eflornithine course were sacrificed in groups of five every 7 days after the Berenil treatment.

#### **2.3.4.2 Azathioprine**

Female NIH mice were infected with  $4 \times 10^4$  trypanosomes of the eflornithine-sensitive *T.b. brucei* stabilate GVR 35/C1.5. They were treated with 100mg/kg azathioprine on day 24 to day 32 after infection and a single Berenil injection (40mg/kg) on day 26 post infection (Table 2.3). At this dosage, azathioprine has been reported to prevent and ameliorate inflammatory cell infiltration into the brains of *T.b. brucei*-infected mice (Hunter, Jennings Kennedy and Murray, 1992b). Higher doses of azathioprine were not well tolerated, with mice dying early in the course of treatment (F.W. Jennings, personal communication), and lower doses were not effective (personal observations). Mice were sacrificed at the end of azathioprine course and every 7 days, thereafter.

### **2.3.5 Evaluation of the effect of eflornithine and azathioprine on astrocyte activation during an established post-treatment encephalitis**

#### **2.3.5.1 Eflornithine**

Female NIH mice infected with the eflornithine resistant *T.b. brucei* trypanosome stabilate and divided into two groups (Table 2.4). The first group (E5) was treated with 40mg/kg Berenil i.p. on day 21 post infection. Seven days after the Berenil injection, they received 2% eflornithine *ad libitum* in drinking

**Table 2.4**  
**Treatment schedules for evaluation of the effect of eflornithine and putrescine on an established post-treatment reactive encephalitis**

E5	I	0	EEEEEEEEEEEE					
			B	K	K	K	K	
			21	28	35	42	49	
							56	
E6	I	0	EEEEEEEEEEEE					
			B	K	K	K	K	
			28	35	42	49	56	
							63	
							70	
							77	
EP	I	0	PPPPPPPPPPPP					
			B	K	K	K	K	
			28	35	42	49	56	
							63	
							70	
PC	I	0	PPPPPPPPPPPP					
			B	K	K	K	K	
			28	35	42	49	56	
							63	
							70	
							7	

The table illustrates the treatment schedules for evaluating the effect of eflornithine (E) and putrescine (P) on PTRE in mice infected with the eflornithine-resistant *T.b. brucei* stabilate. Groups E5 and E6 were treated with eflornithine 7 days after the first (E5) and the second (E6) Berenil treatment. Putrescine was administered simultaneously with eflornithine for 14 days starting from 7 days after the second Berenil treatment (Group EP). PC represent the putrescine treated control group of mice. I = infection, B = Berenil treatment, E = eflornithine treatment, P = putrescine treatment, K = killing time point. Numbers represent time in days after infection

water for 7 or 14 days.

The second group, E6, was injected with 40mg/kg Berenil i.p., on day 28 after infection and again after a relapse of parasitaemia (20-22 days after the first Berenil). This was followed, 7 days later, by administration of 2% eflornithine *ad libitum* in drinking water for 7 or 14 days. During eflornithine therapy animals were maintained in a 2-hour light, 4-hour dark environment to ensure an even intake of eflornithine (Gillet *et al*, 1986).

Five mice were sacrificed on the last day of eflornithine treatment and 7 days (group E6) or 7 and 14 days (group E5), thereafter.

#### **2.3.5.2 Eflornithine and putrescine**

A group of NIH mice were infected with the eflornithine resistant *T.b. brucei* stabilate GVR 35/C1.3 DFMO 5. They were treated with 40mg/kg Berenil on day 21 after infection and again after relapse. They were then divided into 2 groups (Table 2.4). Seven days after the second Berenil injection, the first group of mice (EP) were treated with a combination of 2% eflornithine in drinking water and 400mg/kg/day putrescine i.p. for 14 days. The second group of mice (PC) received putrescine, for 14 days starting from 7 days after the second Berenil treatment. Mice were then sacrificed on the last day of eflornithine + putrescine administration.

#### **2.3.5.3 Azathioprine**

Female NIH mice, infected with  $4 \times 10^4$  trypanosomes of the eflornithine sensitive *T.b. brucei* stabilate, were treated with 40mg/kg Berenil on day 26 after infection. They were then divided into two groups (Table 2.5). One group, A2, was treated with 100mg/kg azathioprine for 7 or 14 days starting from day 32 after infection (6 days after induction of PTRE). Mice were sacrificed, in groups of 5, on the last day of azathioprine administration.



The second group of mice, A3, was treated with 40mg/kg Berenil after relapse of parasitaemia, and with 100mg/kg azathioprine for 7 or 14 from the day of the second Berenil treatment for 7 or 14 days. Five mice were sacrificed at the end of the azathioprine therapy and every 7 days thereafter.

## **2.4 EVALUATION OF PATHOLOGICAL CHANGES IN THE BRAIN**

### **2.4.1 Perfusion of the brain to remove peripheral blood**

At sacrifice, mice were perfused with normal saline (0.9% NaCl) pH 7.0, to remove peripheral blood from the brain. The mice were terminally anaesthetised with trichloroethylene (BDH, analytical grade) and while the heart was still beating, the chest cavity was opened at the level of xiphoid cartilage to expose the heart. The right atrium was perforated to create an outlet for the blood plus saline in order to facilitate the perfusion. Normal saline was injected into the left ventricle with a hypodermic, gauge 20 needle. Approximately 150ml saline was used to achieve sufficient perfusion. The clearance of blood from the kidneys was used as an indication of sufficient perfusion.

### **2.4.2 Fixation of the brain for histopathology and immunocytochemistry**

The perfused brain was removed and fixed in 10% neutral buffered formaldehyde for at least 2 hours and then post-fixed in corrosive formol for a minimum of 24 hours. The embedding of the brains in wax was preceded by a series of dehydrations with methylated spirit and absolute alcohol. All the stages were carried out in a programmable machine under vacuum and pressure. Thus the brains were put through three, 1-hour cycles in methylated spirit, followed by three, 1-hour cycles in absolute alcohol at 40°C. This was followed by a 1-hour clearing in xylene/alcohol (1:1) and another, 1-hour cycle in xylene at 40°C. The brains were then put through four cycles of wax processing at 60°C, the first two of 1 hour each followed by two, 2-hour cycles.

Transverse (coronal) sections, 3-4 $\mu$ m thick, were made through the forebrain, midbrain and hindbrain and stained with haematoxylin and eosin.

Mercuric chloride-formalin (Corrosive formol or Formal sublimate)

Saturated aqueous mercuric chloride	900ml
Formalin	100ml

Neutral Buffered formalin (10%)

Formalin	100ml
Acid sodium phosphate monohydrate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ )	4g
Anhydrous disodium phosphate ( $\text{Na}_2\text{HPO}_4$ )	6.5g
Make up to 1 litre with tap water	

### 2.4.3 Immunostaining for evaluation of astrocyte activation

Immunostaining for glial fibrillary acidic protein (GFAP) was used to identify astrocytes and to aid assessment of astrocyte activation in brain sections. Mercuric chloride reacts with diaminobenzidine (DAB) to produce a brown colouration, creating unwanted background staining. To remove the effects of mercuric chloride all sections were washed in Lugols iodine and then with 1% sodium thiosulphate for 5 minutes, with a rinse in water after each wash. The sections were then dewaxed in xylene for 10 minutes and hydrated through decreasing concentrations of alcohol. Thus, the sections were placed in 100% ethanol through 90%, 70% and 50% ethanol to water for 3 minutes at each concentration. Peroxidase activity in tissues was quenched by soaking the sections in 3%  $\text{H}_2\text{O}_2$  for 5 minutes and rinsing in PBS, pH 7.4. Non-specific antibody binding was blocked by incubation with normal swine serum for 30 minutes.

A polyclonal rabbit anti-GFAP antibody (DAKO) diluted 1:1,000 in PBS-0.1% bovine albumin was placed on the sections and left overnight at 4°C. After rinsing in PBS, the sections were incubated with the secondary antibody, swine anti-rabbit immunoglobulins (DAKO) diluted 1:500 in PBS for 30 minutes at room temperature. After washing in PBS (3x5 minutes), the sections were placed in the peroxidase substrate diaminobenzidine (DAB) for 5 minutes to develop colour for visualisation and rinsed in water. The sections were then counter stained with haematoxylin for 10 seconds, rinsed in tap water, blued in Scot's tap water substitute and dehydrated before mounting with DPX mountant (BDH) and coverslips.

Phosphate Buffered Saline

Sodium chloride (KCl)	0.2g
<i>di</i> -Sodium hydrogen orthophosphate (Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O)	2.9g
Potassium dihydrogen orthophosphate (KH <sub>2</sub> PO <sub>4</sub> )	8.0g
Potassium chloride (KCl)	0.2g

Make up to 1 litre with distilled water and adjust to pH 7.4

DAB solution

H <sub>2</sub> O <sub>2</sub>	300µl
PBS	500ml
DAB	100mg

DAB is carcinogenic, so all work with DAB was performed in a safety cabinet (DAB cabinet), with gloved hands and protective clothes, to prevent contact and inhalation. Thirty ml of PBS/H<sub>2</sub>O<sub>2</sub> mixture was aspirated into a syringe and injected into an isopac containing 100mg of DAB powder and

shaken to dissolve it. The mixture was then aspirated out and mixed with the remaining of PBS/H<sub>2</sub>O<sub>2</sub> mixture and used for colour development.

## **2.5 PREPARATION OF *IN VITRO* CULTURES OF ASTROCYTES**

### **2.5.1 Dissection and dissociation of cells**

Astrocyte preparation was performed as described by Wolswijk and Noble (1989). Two to 3-day-old mice were decapitated and the heads were transferred to a sterile tissue culture room where they were sprayed with 70% ethanol. The skull was exposed by removing the skin, cutting from the occiput and just below the left ear, along the temple to above the left eye and then round above the right eye, along the temple, below the right ear and back to the occiput. Using a separate sterile set of instruments, the crown of the skull was removed along the same cutting lines as the skin to expose the brain.

The cerebral cortices were removed by gently teasing them away from the cerebellum and the hindbrain along the pineal recess, and gently lifting them away from the brain stem. They were then placed upside down in a sterile petri dish containing Hank's balanced salt solution (HBSS). The hippocampus was removed by gentle dissection along the extension of the lateral ventricles. The cortices were then turned upside up and the meninges removed, leaving them free of blood vessels. The "clean" cortices were transferred to another HBSS-containing sterile dish.

To dissociate the cells, the cortices were removed from the HBSS-containing-petri dish, placed onto another sterile dry petri dish and chopped into small pieces using a scalpel blade. The pieces were incubated with collagenase for 45 minutes at 37°C, centrifuged at 89.6g for 10 minutes and the supernatant discarded. They were then incubated with 0.125% trypsin (Bovine pancreas type III, Sigma), in ethylene diamine tetra acetic acid (EDTA), for 30 minutes at 37°C. A mixture of soya bean trypsin inhibitor and DNase was used to inactivate trypsin and digest any free DNA. The cells were centrifuged again at

89.6g for 10 minutes and the supernatant was removed. A series of triturations, first in a 5-ml pipette and then through a 23 gauge-needle were carried out. The cells were washed in 10ml of HBSS, by spinning at 89.6g, after each trituration and then removing the supernatant. The cells were resuspended in culture medium and counted in an improved Neubauer haemocytometer.

The dissociated brain cells were plated at a density of  $5 \times 10^6$  in poly-L-lysine- (PLL-) coated, 80cm<sup>2</sup> tissue culture flasks (Nunclon). They were grown in Dulbecco's modified Eagles medium containing 1.0g/l glucose and supplemented with 10% fetal calf serum (DMEM/10%FCS,) (Gibco), 2mM glutamine (Sigma) and 25 $\mu$ g/ml gentamycin (Flow Laboratories). Once a confluent monolayer of flat cells had been formed (about 7-10 days), the layer of process bearing cells growing on the flat cells were removed by treating with 20 $\mu$ M cytosine arabinoside (AraC) (Sigma) for 48 hours to eliminate most of the faster dividing non-astrocytes.

### **2.5.2. Purification of the cell cultures**

The cells were removed from the flask by washing with Ca<sup>2+</sup> Mg<sup>2+</sup> free Hanks balanced salt solution (HBSS) (Gibco), followed by an incubation in 0.54mM EDTA (Sigma) in HBSS for 10-15 minutes, and the addition of trypsin (Bovine pancreas type III) (Sigma) to a final concentration of 300 I.U./ml. After the cells had detached from the surface of the flask, the trypsinisation was stopped by adding 1ml per 10ml of EDTA/trypsin solution of SBTI-DNase [5200 I.U. Soybean trypsin inhibitor (Sigma) and 3.0mg/ml bovine serum albumin (BSA fraction V, Sigma) in DMEM]. Cells were then slowly trituated through a 10ml-blow out pipette, centrifuged for 10 minutes at 89.6g, resuspended and treated in suspension with rabbit complement (Buxted Ltd, diluted 1:10) plus antibody A2B5 (ascites 1:1,000, Eisenbarth, Walsh and Nirenberg, 1979), monoclonal antibody O4 (concentrated hybridoma supernatant

1:100, Sommer and Schachner, 1981) and anti-galactocerebroside (GalC) monoclonal antibody (hybridoma supernatant 1:10, Ranscht, Clapshaw, Price, Nobel and Seifert, 1982). Treatment with these antibodies eliminated A2B5<sup>+</sup> neurones, A2B5<sup>+</sup>, O4<sup>-</sup>, GalC<sup>-</sup> oligodendrocyte type 2 astrocyte-progenitor cells, A2B5<sup>+</sup> GFAP<sup>+</sup>, type 2 astrocytes, and GalC<sup>+</sup> oligodendrocytes (Abney, Williams and Raff, 1983; Raff, Abney, Cohen, Lindsay and Nobel, 1983a; Raff, Miller and Nobel, 1983b, Sommer, Lagenaur and Schachner, 1982). The remaining cells were plated on PLL- coated flasks at a density of 5x10<sup>6</sup> cell per flask and maintained in 50% fresh DMEM 10%FCS + 50% astrocyte conditioned medium (ACM) (as in Nobel and Murray, 1984). Cultures were kept in an humidified incubator (Flow Laboratories) at 37°C and 92.5% air and 7.2% CO<sub>2</sub>. At this stage, over 95% of the cells in these cultures should have the antigenic phenotype of type-1 astrocytes (GFAP<sup>+</sup>, A2B5<sup>-</sup>) as determined by indirect immunofluorescence (Raff *et al*, 1983a). In my experiments, using immunofluorescence, over 3000 cells were counted and 99.8% were type-1 astrocytes.

#### Dulbeccos modified Eagles medium

Low glucose form of DMEM was purchased from Gibco

#### DMEM +10% Fetal Calf Serum

FCS (Gibco)	10ml
DMEM (Gibco)	90ml
Glutamine (200mM)	1ml
Store at 4°C.	

#### Astrocyte conditioned medium

Culture supernatant collected from a culture of pure astrocytes after at least 24 hours on the cells. It contains growth factors

secreted by astrocytes.

Hanks Balanced Salt Solution

Ca<sup>++</sup> and Mg<sup>++</sup> free (Gibco)

Leibovitz Medium (L15)

From Gibco

Collagenase

Collagenase type II from Worthingtons (200U/mg)

A working solution of 2000U/ml was made by dissolving  
10mg/ml Collagenase in L15

Trypsin

Bovine pancreas trypsin from Sigma

A stock solution of 2.5mg/ml was made by dissolving trypsin in  
HBSS. It was then filtered through 0.22µm filter, and stored in  
1 and 2ml aliquots.

and stored at -20°C. Before use, the trypsin was diluted 1:1  
with EDTA

Soya bean trypsin-inhibitor-DNAse (SBTI/SD)

Dissolved the following in L15

Bovine serum albumin

fraction V (Sigma, U.K.) 3.0mg/ml

Soybean trypsin inhibitor (Sigma) 0.52mg/ml

Bovine pancreas DNAse (Sigma) 0.04mg/ml

The mixture was placed on a roller mixer and left dissolving for 20 minutes.

The solution was then passed through 0.22 $\mu$ m pore filter and stored in 2ml aliquotes at -70°C.

#### Ethylene diamine tetracetic acid (EDTA)

A stock solution was made by dissolving 0.2mg/ml EDTA (Sigma) in Ca<sup>++</sup> Mg<sup>++</sup> free HBSS (Gibco). It was then filtered through 0.22 $\mu$ m pore filter and stored in 20ml aliquotes at 4°C.

#### Glutamine

200mM sterile solution purchased from Sigma, was aliquoted into 5ml portions and stored at -70°C.

#### Poly-L-lysine (PLL)

25mg vials were purchased from Sigma. Stock solutions of 4mg/ml were made, filtered through 0.22 $\mu$ m, aliquoted into 300 $\mu$ l portions and stored below -20°C. At the time of use, the 300 $\mu$ l aliquotes were made up to 40ml with double-distilled, water and used to coat the flasks.

### **2.5.3 Evaluation of the purity of the cell cultures**

A flask containing a confluent monolayer of astrocytes was trypsinised and cells collected in a centrifuge tube. The trypsin was inactivated with soya bean trypsin inhibitor, the tubes centrifuged at 89.6g and supernatant discarded. The cells were then washed twice in HBSS, i.e., the cells were resuspended in HBSS, centrifuged again and supernatant discarded twice. The cells were resuspended in DMEM/10%FCS, counted and seeded onto 13mm-diameter, PLL-coated coverslips in a 24-well tissue culture plate (Nunclon), at a density of 4x10<sup>4</sup> cells per coverslip. They were then maintained in 50% DMEM/10%FCS and 50% astrocyte conditioned medium for 24 hours, in 92.5% air, 7.2% CO<sub>2</sub>

in a humidified incubator, at 37°C.

The cells were stained with fluorescein-labelled rabbit anti-GFAP as follows: the coverslips were removed from the wells and washed three times in staining solution. To wash the cells a coverslip was held with tissue forceps and dipped into three Bijou bottles containing fresh staining solution and gently moved up and down for 10 seconds in each bottle. The coverslips were then placed on mounds in a staining tray and kept moist with a film of staining solution. The cells were incubated with 50µl/coverslip of fluorescein-labelled rabbit anti-GFAP antibody diluted 1 in 1,000 in staining solution, for 30 minutes at room temperature. The antibody solution was drained by tipping the edge of the coverslip onto a tissue paper followed by washing the cells three times, in staining solution, as before. The cells were chilled at -20°C for 10 seconds, fixed with 50µl/coverslip prechilled methanol for 10 minutes at -20°C and washed in distilled water until the methanol streaks disappeared. The coverslips were then mounted onto slides (with the cells facing the slide) with Citifluor and the edges sealed with glyceel.

Under a fluorescent microscope, the GFAP<sup>+</sup> cells were fluorescent and the GFAP<sup>-</sup> cells were non-fluorescent. The cells were counted and the percentage purity calculated as follows:

$$\frac{\text{number of fluorescent cells}}{\text{total cells counted}} \times 100$$

Over 3000 cells were counted and 99.8% were GFAP<sup>+</sup>.

#### Staining solution

10x HBSS (Gibco)	50ml
Donor calf serum	25ml
10% sodium azide (in PBS)	5ml

HEPES (Sigma)	2.38g
HEPES sodium salt (Sigma)	2.60g
Make up to 500mls with distilled water and store at 4°C	

## 2.6 PREPARATION OF ASTROCYTE STIMULI

### 2.6.1 Separation of trypanosomes from host blood cells

Four to 5 days after infection with *T.b.brucei* stabilate GVR 35/C1.5, mice developed parasitaemia ranging from  $10^7$  to  $10^{10}$  trypanosomes per ml of blood. They were bled by cardiac puncture with 26G x 1/2inch needle and a 2ml-syringe, containing a drop of heparin, under terminal anaesthesia. The blood from 5 such mice, was dispensed into a tube and placed on ice till trypanosomes could be separated.

Mouse blood cells carry a more negative charge than do trypanosomes and so can be adsorbed to the anion-exchange diethylaminoethyl (DEAE)-cellulose column, while trypanosomes pass through, retaining viability and infectivity (Lanham and Godfrey, 1970). The process is efficient with salivarian trypanosomes, but less so with stercorarian trypanosomes because of the smaller difference of surface charge between host cells and the organisms of this latter group.

Buffers were prepared from analytical grade reagents and included phosphate-saline glucose buffer (PBGS) pH 8.0. The anion exchanger DEAE-cellulose (Type DE 52, Whatman, Chromedia) in conjunction with PBGS have been reported to give the best yields of all trypanosomes (Lanham, 1968) and was therefore used in this study. The DEAE-cellulose was equilibrated with batches of PBGS buffer in the ratio of 100g of preswollen adsorbent (DE 52 as marketed) to 1.5l. The adsorbent was allowed to settle for about 20 minutes and the supernatant, containing the fine grains of cellulose, was sucked off. This was repeated six times when the pH of the supernatant was within 0.05 units of pH 8.0 (the PBGS pH).

The preparation of the column and fractionation of blood were carried out at room temperature, although the unprocessed blood and the eluate were kept on ice. Columns ranging from 0.4 to 2.5cm in diameter and 4-8cm in height are satisfactory for fractionating up to 5ml of blood (Lanham, 1968). For this reason, the column used for the 3-7ml of blood from 5 infected mice in this study was packed into a 50ml-syringe, which is 3cm in diameter, to a height of about 10cm. To pack the column, the plunger was removed from a 50ml-hypodermic syringe, the lower end of the syringe was packed with glass wool and a rubber tubing with a clip, to control flow rate, attached to the outlet. The apparatus was clamped onto a retort stand and receivers for eluate placed in an ice bath at the bottom. The glass wool was moistened with PBGS and the slurry of equilibrated DEAE-cellulose poured in. The excess liquid was drained and the eluating buffer (PBGS) was run through the column to bed down the adsorbent with a firm horizontal surface, and to remove any air bubbles from the system. The outlet was closed and cold blood carefully layered on to the surface. A rate of flow was maintained, using gentle suction when necessary, to give a sharp descending front of erythrocytes. When all the blood had entered the adsorbent, buffer was run through the column until microscopic examination showed that few or no trypanosomes were still being eluted. The trypanosomes were separated from the eluate by centrifugation at 4°C for 20 minutes at 1,630g, followed by washing with buffer to remove the plasma proteins. Trypanosomes were either used directly for stimulating astrocytes or pelleted and stored in liquid nitrogen for future use.

Phosphate-saline glucose buffer

0.2M Na <sub>2</sub> HPO <sub>4</sub>	385ml
0.2M NaH <sub>2</sub> PO <sub>4</sub>	15ml
0.85 % (w/v) NaCl	300ml

2.5% (w/v) Glucose	400ml
Adjust to pH 8.0	

Diethylaminoethyl cellulose

DEAE cellulose ( DE 52 Whatman)	100g
Phosphate-saline glucose buffer	1.5l

### **2.6.2 Preparation of whole trypanosome lysate**

Trypanosome pellet stored in liquid nitrogen was repeatedly freeze-thawed by plunging into liquid nitrogen, and thawing at room temperature. The crude lysate was then sonicated at 4°C for 2hrs using a bath sonicator. Protein concentration was measured using the Biorad protein assay method (Bradford, 1976).

### **2.6.3 Quantification of the protein content in the trypanosome lysate**

Bovine serum albumin, supplied by Biorad with the protein assay reagent, was reconstituted and serially diluted to make concentrations ranging from 0.2 to 1.4mg/ml. The dye reagent, phosphoric acid and methanol was diluted 1 in 5 to make a working dilution. The standards or test samples were mixed with the dye reagent at 1 in 50 dilution. Optical density for each of the standard dilutions was read at OD<sub>595</sub> against the Biorad-reagent blank, and a standard curve plotted. The optical density for the trypanosome lysate was determined and the concentration extrapolated from the standard curve.

### **2.6.4 Purification of the membrane-bound form of the trypanosomal variable surface glycoprotein**

Purification of membrane-bound variable surface glycoprotein (VSGm) was carried out as described by Jackson, Owen and Voorheis (1985). Dr. Joanne Burke of the Department of Veterinary Medicine, extracted and

determined the purity of the first VSGm, used in this study. This technique is based on two facts: 1, that the radiolabelled VSGm, after treatment with trichloroacetic acid, partitions along with several other proteins into chloroform/methanol, as long as the molar fraction of water is low enough to give a single-phase system; and 2, that when the organic extract is separated into two phases, by addition of aqueous NaCl, the VSG alone re-partitions into the aqueous phase (Jackson *et al*, 1985).

A suspension of bloodstream forms of *T.b. brucei* stabilate GVR 35/C1.5 ( $1 \times 10^{10}$  to  $5 \times 10^{10}$ ) in 20mls of Krebs-Ringer phosphate buffer (Voorheis, 1980) was treated with an equal volume of 10% (w/v) trichloroacetic acid at 0°C and the precipitate centrifuged at 9000g for 10 seconds. It was critical to obtain a loose pellet at this stage. The pellet was resuspended in distilled deionised water ( $4 \text{ml}/10^{10}$  cells) with the aid of a 1ml pipette. The suspension was extracted with 20 volumes of chloroform/methanol (2:1 v/v) by vigorous shaking for 5 minutes followed by storage overnight at 5°C. This storage procedure increases the final yield of purified VSGm. The extract was then separated into two phases by addition of 0.2 volumes of 0.9% NaCl solution, and was centrifuged at 12,000g for one hour. The upper, aqueous phase contained pure VSGm, and was removed by aspiration and dialysed against a total of 60l of distilled deionised water at 4°C over a duration of 36 hours, with 15 changes of distilled water. Thus, during the first 8 hours of dialysis, the distilled water was changed every hour. Thereafter, the water was changed every 4 hours. The retained material was freeze dried and the resulting VSG stored dry over  $\text{P}_2\text{O}_5$  *in vacuo* until required. Only 50% of VSGm dissolves in distilled deionised water. To dissolve it completely, 1M NaOH was added to the VSGm suspension to bring pH to 10.0 followed, immediately, by neutralisation with 1M Tris/HCl. A separate titration to determine the volume of Tris required for complete neutralisation of the acid was performed and

worked out to be 1:400 NaOH:Tris. The final extract was subjected to SDS/polyacrylamide gel electrophoresis (Studier, 1972), followed by staining with Coomassie blue. This revealed a major polypeptide of approximately 58kD, which corresponds to the molecular weight of VSGm.

#### Krebs-Ringer phosphate buffer

NaH <sub>2</sub> PO <sub>4</sub>	2mM
Na <sub>2</sub> HPO <sub>4</sub>	20mM
NaCl	98mM
MgCl <sub>2</sub>	1mM
KCl	2mM
D-glucose	10mM

#### **2.6.5 Lipopolysaccharide**

The lipopolysaccharide (LPS) used in our experiment was derived from *Escherichia coli* serotype 026:B6 (Sigma, 2% solution).

#### **2.6.6 Drugs**

The drugs used in this study were eflornithine (difluoromethyl ornithine, DFMO) a gift from Dr F.W. Jennings, originally obtained from Merrell Dow Institute, and azathioprine, purchased from Wellcome Laboratories.

#### **2.6.7 Exposing astrocytes to the stimuli**

LPS is known to induce production of various cytokines when applied to cultures of astrocytes and was therefore used as a positive control in our astrocyte cultures. The cell supernatants were removed under aseptic conditions and replaced with fresh medium containing the various stimuli: 10µg/ml LPS; 25µg/ml and 50µg/ml whole trypanosome lysate; 25µg/ml VSG; 10<sup>4</sup> and 10<sup>7</sup>

live trypanosomes/ml. The cells were then incubated at 37°C, 7.2% CO<sub>2</sub> and 92.5% air in a humidified incubator. Flasks of cells were harvested at 2, 4, 6 and 8 hours with an additional 24-hour harvest for the group of flasks that were exposed to live trypanosomes. Fresh culture medium was used in control flasks to establish baseline values.

To evaluate the effect of eflornithine and azathioprine on LPS-stimulation of astrocytes, 0.5mM and 5mM eflornithine, or 10µg/ml azathioprine were added simultaneously with 10µg/ml LPS to flasks containing confluent monolayers of astrocytes. Astrocytes were harvested, as described above, at the various experimental intervals (2, 4, 6 and 8 hours) after addition of these stimuli and processed for RNA extraction.

## **2.7 EVALUATION OF CYTOKINE GENE EXPRESSION**

### **2.7.1 Extraction of RNA from the cell cultures**

RNA was prepared as described by Chomczynski and Sacchi (1987), using acid-guanidinium-thiocyanate-phenol-chloroform extraction. This method is a single-step extraction that permits recovery of total RNA from small quantities of tissue or cells making it suitable for gene expression studies for which only a limited quantity of biological material is available.

At the end of each stimulation, the supernatant was removed and the astrocytes were washed twice with Ca<sup>2+</sup> Mg<sup>2+</sup> free HBSS. This was replaced with 1ml denaturing solution which was spread over the cells using a rubber policeman. The cells were scraped off the flask and transferred into 10ml polypropylene tubes and homogenised using a 1ml syringe and a gauge 25 needle. Sequentially, 0.1ml of 2M sodium acetate, pH 4, 1ml phenol (water saturated), and 0.2ml chloroform/isoamyl alcohol mixture (49:1) were added to the homogenate with thorough mixing by vortexing after addition of each reagent. The final mixing was a 10 second vortex followed by cooling on ice

for 15 minutes. Samples were centrifuged at 12,000g for 20 minutes at 4°C after which the RNA in the aqueous (upper) phase was transferred into a fresh polypropylene tube. RNA was precipitated by addition of 1ml of cold isopropanol and incubation on dry ice for 20 minutes, followed by sedimentation by centrifugation at 12,000g for 20 minutes at 4°C. The resulting pellet was dissolved in 0.3ml of denaturing solution and transferred into a 1.5ml Eppendorf tube, and precipitated with 2 volumes of ethanol (analytical grade) on dry ice for 30 minutes. After centrifugation in a microfuge for 10 minutes at 4°C, the RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried for 15 minutes and dissolved in 50µl of distilled deionised water and stored at -20°C until required.

#### Denaturing solution

Guanidinium thiocyanate	4M
Sodium citrate	25mM, pH 7
N-Lauryl-sarcosine	0.5%
2-β-mercaptoethanol	0.1M

#### **2.7.2 Quantification of the RNA**

The RNA extracted was quantified by measuring the optical density in a spectrophotometer. Thus, 10µl of RNA sample was made up to 1ml with sterile water and placed in a quartz cuvet (1 in 100 dilution). The spectrophotometer was zeroed with water and then used to determine the optical density of the RNA samples. The optical density at 260nm and at 280nm was read against water. The ratio  $OD_{280}/OD_{260}$  should be 1.8 for pure RNA. For the purpose of this study a ratio of greater than 1.5 was acceptable. The concentration of RNA was worked out by multiplying with a correction factor of 40 and the dilution factor (Sambrook, Fritsch and Maniatis, 1989) to give the concentration in µg/ml.

### 2.7.3 Generation of complementary DNA and amplification by polymerase chain reaction

This involves the generation of complementary DNA (cDNA) from mRNA by reverse transcription, followed by amplification of the resultant cDNA by a polymerase catalysed chain reaction (PCR). PCR uses a thermostable DNA polymerase and specific oligonucleotide primers to conduct repeated cycles of DNA synthesis, *in vitro*, on a very small amount of template DNA (Mullis and Faloona, 1987; Saiki, Gelfand, Stoffel, Scharf, Hiruchi, Horn, Mullis and Erlich, 1988). Using this procedure, a specific segment of template DNA can be amplified over a million times making the subsequent detection of that segment easier. The thermostable DNA polymerase was purified from the thermophilic bacterium, *Thermus aquaticus* and named *Taq* DNA polymerase (Chien, Edgar and Trela, 1976). This enzyme can survive extended incubation at 95°C and therefore is not inactivated by the heat-denaturation step of PCR. In addition, because annealing and extension of oligonucleotides can be carried out at elevated temperatures, mispriming is greatly reduced.

This study screened for transcripts for the cytokines: IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF $\alpha$ , MIP-1 and GM-CSF. The sequences for the oligonucleotide primers for these cytokines are shown in Table 2.6. Approximately 5 $\mu$ g of total RNA was lyophilised and resuspended in 9 $\mu$ l of annealing buffer with 0.5 $\mu$ g of the 3' primer, heated at 83°C for 3 minutes, incubated at 69°C for 20 minutes followed by a 40-minute cooling to 40°C. Fifteen microlitres of cDNA/deoxyribonucleoside triphosphate (dNTP) buffer were added with 200 units of Moloney murine leukaemia virus reverse transcriptase (Gibco) and incubated at 43°C for 45 minutes. For amplification of the cDNA generated by the reverse transcription, 0.5 $\mu$ g of 5' primer was added with 55 $\mu$ l of *Taq* polymerase buffer, 20 $\mu$ l 2.5mM dNTPs and 2 units of DNA polymerase (Promega).

**Table 2.6****Nucleotide sequences 5' to 3' for the murine oligonucleotides used in this study.**

Cytokine	Primer	sequence
$\beta$ -Actin	5'	GTGGGCCGCTCTAGGCACCAA
	3'	CTCTTTGATGTCACGCACGATTTC
IL-1 $\alpha$	5'	ATGGCCAAAGTTCCTGACTTGTTT
	3'	CCTTCAGCAACACGGGCTGGTC
IL-1 $\beta$	5'	ATGGCAACTGTTCTGAACTCAACT
	3'	CAGGACAGGTATAGATTCTTTCCTTT
IL-6	5'	ATGAAGTTCCTCTCTGCAAGAGACT
	3'	CACTAGGTTTGCCGAGTAGATCTC
TNF $\alpha$	5'	ATGAGCACAGAAAGCATGATCCGC
	3'	CCAAAGTAGACCTGCCCGGACTC
GM-CSF	5'	TGTGGTCTACAGCCTCTCAGCAC
	3'	CAAAGGGGATATCAGTCAGAAAGGT
MIP-1	5'	ATGAAGGTCTCCACCACTGCC
	3'	TCAGGCAATCAGTTCCAGGTCAGTGATGTATTC

The mixture was then overlain with 100 $\mu$ l of mineral oil and subjected to 35 PCR cycles of 94°C denaturation for 1 minute, 60°C annealing for 2 minutes and 72°C extension for 3 minutes. Thirty five cycles were chosen because, 2kb in length can be obtained from 30-35 cycles of amplification with only 10<sup>-6</sup> $\mu$ g of starting DNA (Sambrook *et al*, 1989). In addition, under normal reaction conditions, the amount of *Taq* polymerase becomes limiting after an amplification of level of approximately 10<sup>6</sup> has been attained (Sambrook *et al*, 1989).

To visualise the PCR products, 20 $\mu$ l of the reaction mixture was electrophoresed on 2% agarose gel and stained with ethidium bromide.

#### Annealing buffer

KCl	250mM
Tris HCl, pH 8.3	10mM
EDTA	1mM

#### cDNA/dNTP buffer

Tris HCl pH 8.3	24mM
MgCl <sub>2</sub>	16mM
Dithiothreitol	18mM
dNTPs	0.4mM

#### Taq polymerase buffer

Tris HCl pH 8.0	100mM
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	30mM
MgCl <sub>2</sub>	3mM
2-mercaptoethanol	10mM

#### 2.7.4 Electrophoresis of the polymerase chain reaction-products

According to Sambrook *et al*, (1989), electrophoresis is the standard method used to separate, identify, and purify DNA fragments. The method is simple, rapid to perform and capable of resolving fragments of DNA that cannot be separated adequately by other procedures, such as density gradient centrifugation. When an electric field is applied across the gel, DNA, which is negatively charged at neutral pH, migrates toward the anode. The rate of migration is determined by various factors, including, the molecular size of the DNA (Fisher and Dingman, 1971; Aaij and Borst, 1972). Larger molecules migrate more slowly because of greater frictional drag and because they travel through the gel pores less efficiently.

Two grams of multipurpose agarose (Boehringer Mannheim) was mixed with 100ml of Tris boric acid/EDTA buffer (TBE), pH 8.3 and heated in a microwave until all the agarose was melted. A gel plate was put on a level surface (judged with a spirit level) and fitted with a comb of appropriate size to make wells. The molten gel, allowed to cool to about 25°C, was poured onto the gel plate and left to set. The set gel was then placed in a tank containing TBE. Twenty  $\mu\text{l}$  of each PCR product were mixed with 5 $\mu\text{l}$  of an inert dye. Orange G dissolved in TBE/glycerol 1:1 (v/v), and carefully put into the gel wells. At least one well in each gel was loaded with 4 $\mu\text{l}$  of a molecular weight marker, the 123 base pair ladder purchased from Gibco. After loading all the wells with the various samples, electrophoresis was carried out at about 56mA for 1 hour 30 minutes. After separation, the gel was stained with ethidium bromide and the separated transcripts identified by their molecular weight. The molecular weight ladder allowed the sizes of the PCR products to be determined.

### Tris boric/EDTA buffer

Tris HCl	10.8g
Boric acid	5.5g
EDTA	0.93g

Made up to 1 litre with distilled water. pH 8.3

## **2.8 EVALUATION OF THE PRESENCE OF CYTOKINE PROTEIN**

### **2.8.1 Detection of secreted cytokines in the astrocyte supernatants**

An enzyme-linked immunosorbent assay, for mouse TNF, was used to test for secreted cytokines in the cell supernatants following exposure to various stimuli. The protocol followed was a slight modification from the one supplied by Pharmigen laboratories, an adaptation from the manual of clinical and laboratory medicine (Rose, Friedman and Fahey, 1986). Briefly, 96-well microtitre plates (Corning easy wash or Dynatech IV) were coated with 50 $\mu$ l/well of purified anti-mouse TNF antibodies at a concentration of 2 $\mu$ g/ml and incubated overnight at 4°C. The following morning, nonspecific binding was blocked by incubation with 50 $\mu$ l/well of 10% FCS in PBS, pH 7.4, for 45 minutes at 37°C. The plates were rinsed three times in PBS containing 0.05% tween 20, followed by two washes of 3 minutes each.

Recombinant mouse tumor necrosis factor (TNF) purchased from Pharmigen was used as the standard cytokine. Ten serial double-dilutions in PBS starting at 80ng/ml were prepared from the stock. The standard- cytokine dilutions and the neat supernatant samples were added (50 $\mu$ l/well) in duplicates, and incubated at 37°C for 2 hours. The plates were then washed in PBS/tween as before, incubated for 1 hour at room temperature with 50 $\mu$ l/well of biotinylated mouse anti-TNF antibody (Pharmigen), at 4 $\mu$ g/ml, and washed again. Streptavidin peroxidase (Serotec), diluted 1 in 1,000 in 1% BSA/PBS/tween 20 was added (75 $\mu$ l/well) and incubated for 1 hour at 37°C and

washed. The peroxidase substrate, 3,3', 5, 5' tetramethylbenzidine (TMB) was added at 100 $\mu$ l/well. Absorbencies were read at OD<sub>630</sub>, after 15 minutes.

#### PBS/tween

sterile PBS	100mls
Tween 20	0.05mls

20ml aliquotes were stored at -20°C

### **2.8.2 Detection of cytokine proteins in cultured astrocytes**

#### **2.8.2.1 Preparation of cultured astrocytes for immunocytochemistry**

A flask containing a confluent monolayer of astrocytes was trypsinised and cells collected in a centrifuge tube. The trypsin was inactivated with soya bean trypsin inhibitor, the tubes centrifuged at 89.6g and supernatant discarded. The cells were then washed twice in HBSS, i.e., the cells were resuspended in HBSS, centrifuged again and supernatant discarded. The cells were resuspended in DMEM/10%FCS, counted and seeded onto 13mm-diameter, PLL-coated coverslips in a 24-well tissue culture plate (Nunclon), at a density of 4x10<sup>4</sup> cells per coverslip. They were then maintained in 50% DMEM/10%FCS and 50% astrocyte conditioned medium 92.5% air, 7.2% CO<sub>2</sub> in a humidified incubator, at 37°C, for 24 hours. The medium was then replaced with DMEM/10%FCS and cells incubated for a further 24 hours. At this time, confluent monolayers of astrocytes were established in all the coverslips and cells were ready for use.

The medium was replaced with 50 $\mu$ g/ml trypanosome lysate in DMEM/10%FCS (1ml/well), and re-incubated. Plates were removed at 2, 4, 6 and 8 hours after addition of stimulant. The medium was removed and the cells were washed twice with HBSS.

The cells were chilled at -20°C for 10 seconds and fixed with methanol (1ml/well) for 10 minutes at -20°C. The methanol was replaced with 70% ethanol and cells stored at 4°C until immunocytochemistry could be performed.

### **2.8.2.2 Immunostaining for cytokine protein detection in cultured astrocytes**

The coverslips were removed from the ethanol-containing wells, air dried and fixed onto microscopic slides with glassbond in the presence of ultra violet light (to activate glassbond). Nonspecific antibody binding was blocked by incubating the cells with 5% BSA and saponin for 10 minutes at room temperature in a humid chamber. Excess BSA was drained off and the cells incubated, for 1 hour, with the primary antibodies diluted 1 in 50 in 2% BSA/Tris buffered saline containing saponin (2% BSA/TBS/saponin). The primary antibodies consisted of: rabbit anti-mouse tumor necrosis factor (Rb $\alpha$ M TNF), rabbit anti-mouse interleukin 1 (Rb $\alpha$ M IL-1) and rat anti-mouse interleukin 6 (Rt $\alpha$ M IL-6). The cells were washed twice (5 minutes each time) with TBS/saponin and incubated with the secondary antibodies (biotinylated goat anti-rabbit immunoglobulins for Rb $\alpha$ M TNF and Rb $\alpha$ M IL-1 and biotinylated rabbit anti-rat immunoglobulins for Rt $\alpha$ M IL-6 diluted 1 in 200 in 2% BSA/TBS/saponin) for 1 hour at room temperature. The cells were washed again and incubated with the avidin biotin complex alkaline phosphatase conjugate for 30 minutes at room temperature in a humid chamber and washed before the development with naphthol/fast red for visualisation.

### **2.8.2.3 Colour development for visualisation**

The cells were washed in TBS and stained with naphthol red in veronal acetate buffer for 10 minutes in the dark. Each wash consisted of two immersions of 5 minutes each into fresh TBS/saponin each time. They were then counterstained with haematoxylin by placing the slides in haematoxylin for 2 seconds, rinsed in tap water and blued in Scot's tap water substitute. Coverslips were then mounted with aquamount.

Tris buffered saline

Tris/HCl	0.05M
Sodium chloride	0.15M pH 7.6

Veronal acetate buffer

0.1M HCl	0.2ml
Veronal acetate solution	5ml

Veronal acetate solution

Sodium (veronal) barbitone	2.9g
Sodium acetate trihydrate	1.9g
Make up to 100ml in distilled water	

To make up Naphthol red solution

Levamisole	3mg
Fast red	6mg
Naphthol	3mg
Veronal acetate buffer	12mls

Solution A:

Levamisole and fast red, dissolved in 4ml veronal acetate buffer

Solution B:

Naphthol AS-TR phosphate dissolved in two to three drops of dimethylformamide.

Solution B was added to solution A and the mixture filtered in the dark at 4°C to give a clear yellow solution, which was used for colour development.

## **CHAPTER 3**

### **ASTROCYTE ACTIVATION DURING *TRYPANOSOMA BRUCEI* *BRUCEI* INFECTION IN MICE**

### 3.1 INTRODUCTION

Astrocyte activation is a prominent characteristic following many types of insult to the central nervous system (Bignami *et al*, 1972; Latov *et al*, 1979; Nathaniel and Nathaniel; 1981; Smith *et al*, 1987; O'Callaghan *et al*, 1990). When activated, the astrocytes increase in numbers, become larger, extend more processes, and significantly increase their cytoplasmic content of glial fibrillary acidic protein (GFAP), an astrocyte specific intermediate filament (Bignami *et al*, 1972). This process is referred to as reactive gliosis and can lead to the formation of dense gliotic scars in the central nervous system (CNS). Infiltration of inflammatory mononuclear cells (lymphocytes, blood-derived macrophages) and an increase in intrinsic brain microglial cells are additional features of many inflammatory conditions of the brain, including, multiple sclerosis (Traugott, Reinherz and Raine, 1983; Traugott, Scheinberg and Raine, 1985; Cuzner, Hayes, Newcombe and Woodroffe, 1988) and traumatic injuries of the brain (Tsuchihashi, Kitamura and Fujita, 1981; Giulian *et al*, 1989). Reactive gliosis and mononuclear cell infiltration are major features of late-stage HAT (Adams *et al*, 1986; Haller *et al*; 1986)

The CNS pathology in mice chronically infected with *T.b. brucei* is characterised by an infiltration of the subarachnoid and the Virchow Robin spaces by the lymphocyte/plasma cell series and macrophages, and by microglial hyperplasia and large reactive astrocytes in the white matter (Murray *et al*, 1974a; 1974b; Jennings *et al*, 1989; Hunter *et al*, 1991). Subcurative treatment of *T.b.brucei* infections of mice after day 21 of infection results in an exacerbation of this lesion (Jennings and Gray, 1983; Jennings *et al*, 1989; Hunter *et al*, 1992a). These lesions closely simulate the CNS lesions of human sleeping sickness and of reactive arsenical encephalopathy cases (Ormerod, 1970; de Raadt, 1984; Adams *et al*, 1986; Haller *et al*, 1986).

This study was carried out to investigate the progress of astrocyte

activation during the course of *T.b. brucei* infection in mice, and following curative and subcurative trypanocidal therapy.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Infection**

Experiments were performed on female NIH mice weighing between 25 and 30g. These animals were infected intraperitoneally with  $4 \times 10^4$  parasites of either the eflornithine-sensitive or the eflornithine-resistance *T.b. brucei*, stabilate GVR 35/C1.5 and GVR 35/C1.3 DFMO 5, respectively (Chapter 2).

### **3.2.2 Infection without treatment**

Adult female NIH mice were infected with either the eflornithine-sensitive or the eflornithine-resistant trypanosome stabilates (above). Five mice from each group were sacrificed every 7 days during the course of infection. These mice are represented as group IC in Table 3.1. Deaths, as a consequence of infection, were common in this group with few animals surviving longer than 52 days. An uninfected, untreated mouse was also killed at each point.

### **3.2.3 Induction of post-treatment encephalitis by treatment with diminazene aceturate**

Two groups of mice were infected with either the eflornithine-sensitive or the eflornithine-resistant *T.b. brucei* stabilate in order to compare the CNS pathology caused by each of the stabilates. On day 21 after infection, all the mice were treated i.p. with 40mg/kg diminazene aceturate (Berenil, Hoechst, Germany). Each of these two groups were further divided into two groups, P1 and P2 (Table 3.1). The first group, P1, was sacrificed serially every 7 days after the Berenil treatment in groups of five. This treatment, with Berenil on day 21 after infection, is known to be subcurative (Jennings and Gray, 1983)

and to induce a post-treatment reactive encephalitis (PTRE) in *T.b. brucei*-infected mice (Jennings *et al*, 1989; Chapter 1).

The second group, P2, was treated with 40mg/kg Berenil i.p. on day 21 after infection and again after relapse of parasitaemia which occurred 20-22 days after the first Berenil injection. Five mice were killed every 7 days thereafter. This treatment regimen has been reported to cause a very severe post-treatment encephalitis (Jennings *et al*, 1989; Chapter 1).

Groups of uninfected mice, which received the same drug treatment as the infected mice, were sacrificed at the same time as the experimental animals. Some infected, treated mice were retained to monitor relapse of parasitaemia. Blood from tail snips was examined every 2 days until relapse of parasitaemia, when the mice were sacrificed.

#### **3.2.4 Curative treatment after induction of post-treatment encephalitis**

Mice in this group, CT (Table 3.1), were infected, with the two trypanosome stabilates as in the above groups, and were treated with Berenil on day 21 after infection and again after relapse of parasitaemia. Seven days after the second dose of Berenil, mice received two consecutive daily treatments of 15mg/kg MK 436 (Merck Institute for Therapeutic Research) in combination with 5mg/kg Mel Cy (Cymelarsen) (Rhone Merieux) i.p. At this dosage, this combination regimen has been shown to be curative for the CNS stages of *T.b. brucei* infections in mice (Jennings, 1991c). Mice were sacrificed in groups of five, 5, 12, 19 and 26 days after this curative therapy.

The pathology in brains of mice from this group was compared to that of mice treated with the double-Berenil regimen. Uninfected mice treated with same drug regimen as the infected mice were used as controls. A group of mice was retained after curative treatment and parasitaemia was monitored, to ensure the treatment was curative.



### **3.2.5 Monitoring infections**

Mice were monitored for parasitaemia by examination of fresh blood from tail snips. At least twenty fields were examined at x400 magnification before samples were designated either positive or negative.

### **3.2.6 Perfusion of the brain to remove peripheral blood**

At death, mice were perfused with 150ml normal saline through the left ventricle to remove peripheral blood. The brains were fixed in neutral buffered formalin and processed for histopathology (Chapter 2).

### **3.2.7 Immunostaining for astrocyte activation**

Three to 4 $\mu$ m transverse sections were cut at the fore-, mid- and hindbrain. Some of the sections were stained for glial fibrillary acidic protein (GFAP) to assess astrocyte activation (Chapter 2), while others were stained with haematoxylin and eosin to assess pathology (Chapter 2).

### **3.2.8 Assessment of astrocyte activation**

The criteria for identifying astrocyte activation was an increase, above that found in normal uninfected mice (baseline), in one or more of the following parameters: the intensity of GFAP staining, the number of GFAP-staining astrocytes, the number, size, and the degree of branching, of the astrocytic cytoplasmic processes and the topographical distribution of astrocytes in the brain parenchyma.

## **3.3 RESULTS**

### **3.3.1 Astrocyte activation in infected untreated mice**

Figure 3.1 was included to illustrate the areas of the cerebrum (3.1a) and cerebellum (3.1b) most commonly referred to in describing astrocyte activation

### **Figure 3.1**

Sections of the cerebrum (a) and the cerebellum (b) from a normal mouse showing the parts of brain most commonly referred to during the description of astrocyte activation, in this study: m = meninges; cc = corpus callosum; hc = hippocampus; dg = dentate gyrus; cf = choroid fissure; numbers = cerebellar lobes (numbering is not in anatomical order); gl = granular layer of the cerebellum; ml = molecular layer of the cerebellum; b = base of the cerebellum. Haematoxylin and eosin. x40



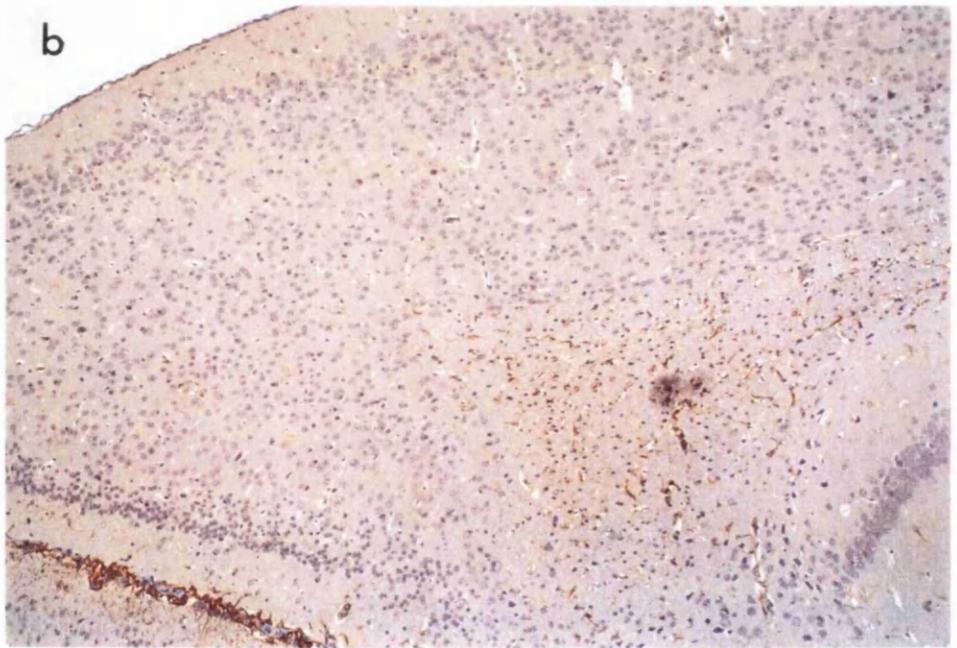
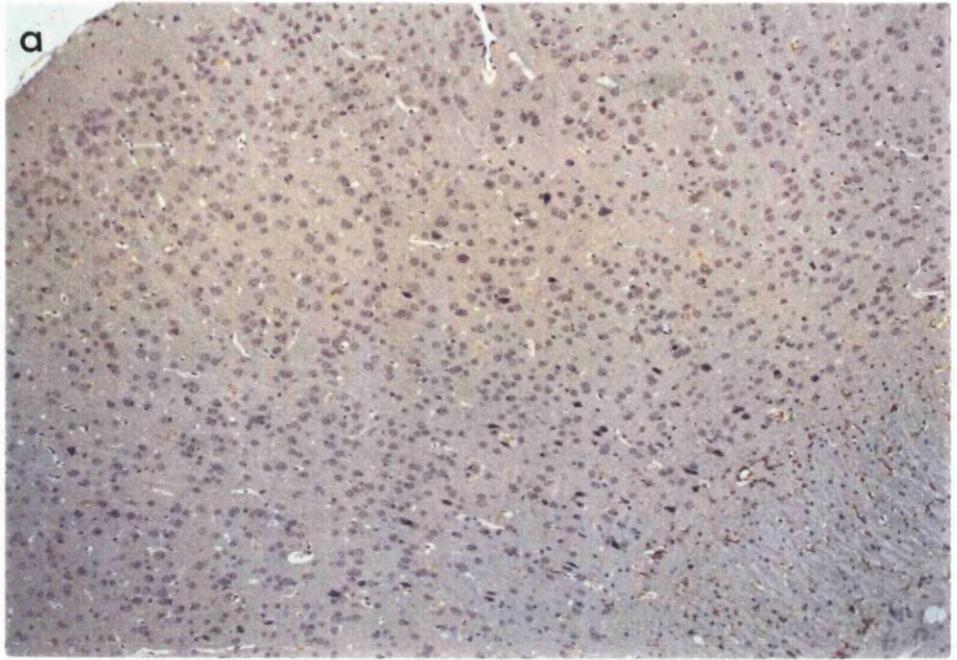
during the course of this study.

In uninfected, untreated mice, very faintly GFAP-staining astrocytes were found along the choroid fissure, around the ventricles, along the major folds such as the superior and inferior sagittal sinuses, the pineal recess and the choroid fissure, in the hippocampus, the corpus callosum and the circumventricular organs, including, the pineal body, area postrema and the median eminence. These astrocytes extended two to four short, non-branched cytoplasmic processes (Figures 3.2a and c). In the cerebellum such astrocytes were found at the base of the cerebellum, bordering the fourth ventricle and the aqueduct, and along the meninges surrounding the base of the cerebellum (Figure 3.2e).

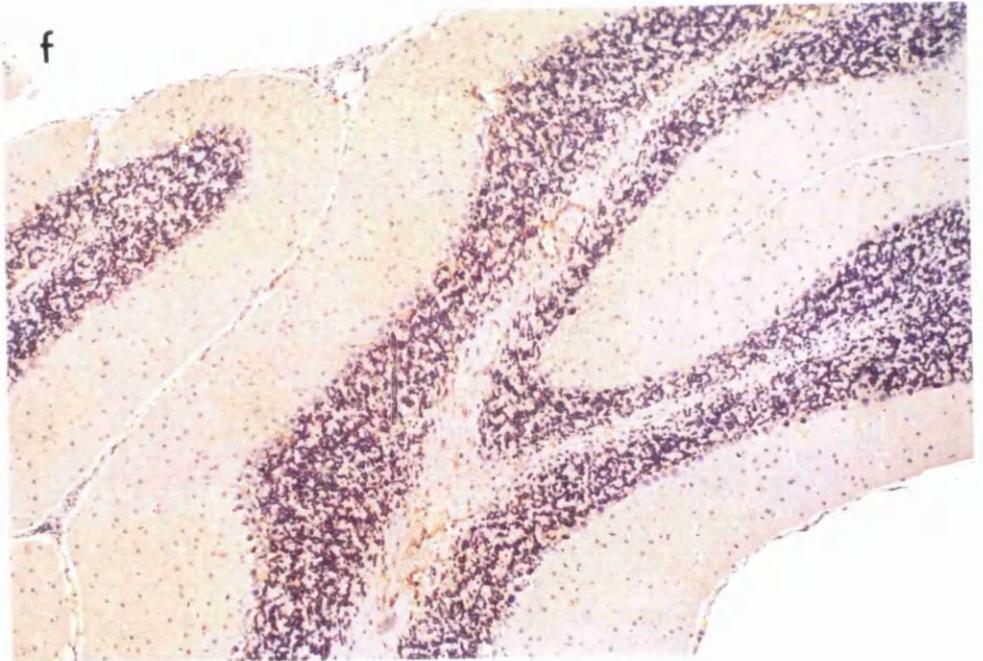
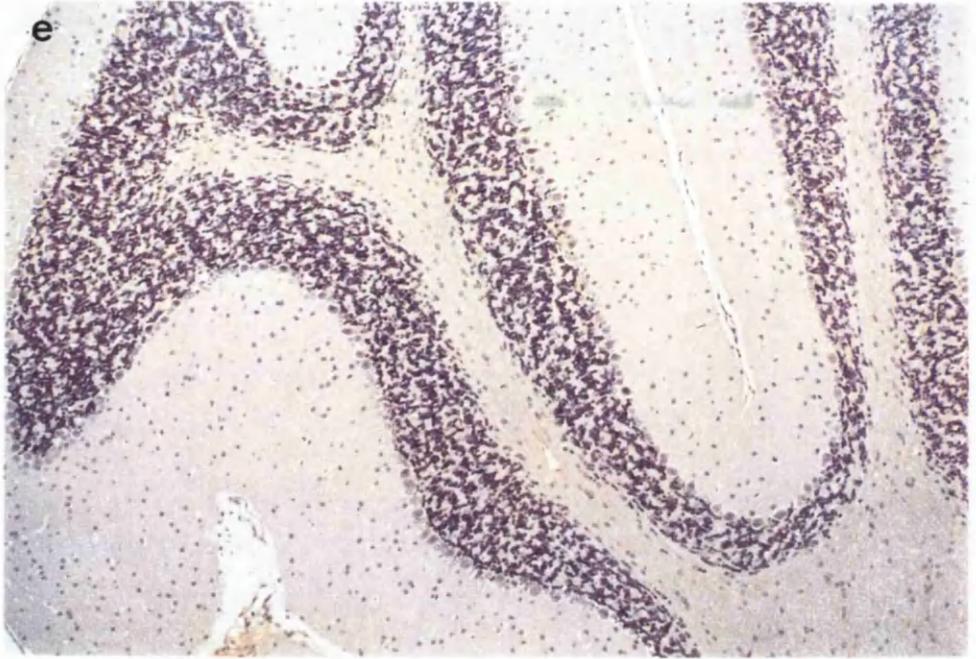
In mice sacrificed 7 and 14 days after infection, the distribution of GFAP-staining astrocytes and the intensity of the staining was comparable to that in normal mice. GFAP-staining astrocytes in mice sacrificed 21 days after infection were similar in morphology and distribution to those in normal animals but the staining intensity was slightly higher. This was taken to be the first sign of astrocyte activation. By the 28<sup>th</sup> day after infection, there was a distinct increase in the intensity of GFAP-staining of astrocytes in the corpus callosum, the hippocampus, along the choroid fissure and around the ventricles, as compared to uninfected controls (Figure 3.2b). The astrocytes in these areas were more in number, appeared larger and extended slightly longer, but still non-branched, cytoplasmic processes compared to the uninfected controls. The astrocytes in the hippocampus extended more (4 or more) processes some of which were branched (Figure 3.2d). In the cerebellum, activated astrocytes were found in the white matter at the base and in the lobes, in the granular layer of the grey matter, along the meninges at the base of the cerebellum, and at the borders of the fourth ventricle and the aqueduct (Figure 3.2f).

### Figure 3.2

Brain sections from uninfected untreated (a, c and e) mice, compared to sections from *T.b. brucei*-infected untreated mice, group IC, sacrificed 28 days post infection (b,d and f). The sections were immunostained with rabbit anti-GFAP (DAKO), developed with DAB/peroxidase kit (Vectastain) and counterstained with haematoxylin. There is an increase in the number astrocytes and their GFAP staining intensity in the corpus callosum (b), and the hippocampus (d). Faintly GFAP-staining astrocytes can be seen around the blood vessels and in the granular layer of the cerebellum of mice sacrificed 28 days after infection (f). Meningitis is very mild in the cerebrum (b) and moderate in the cerebellum (f). x90







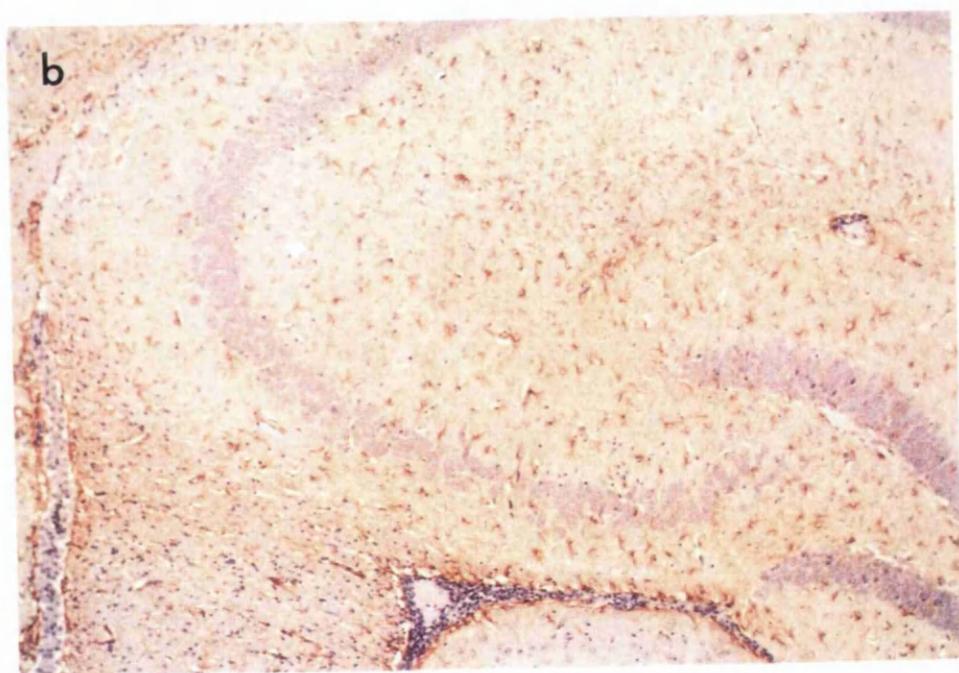
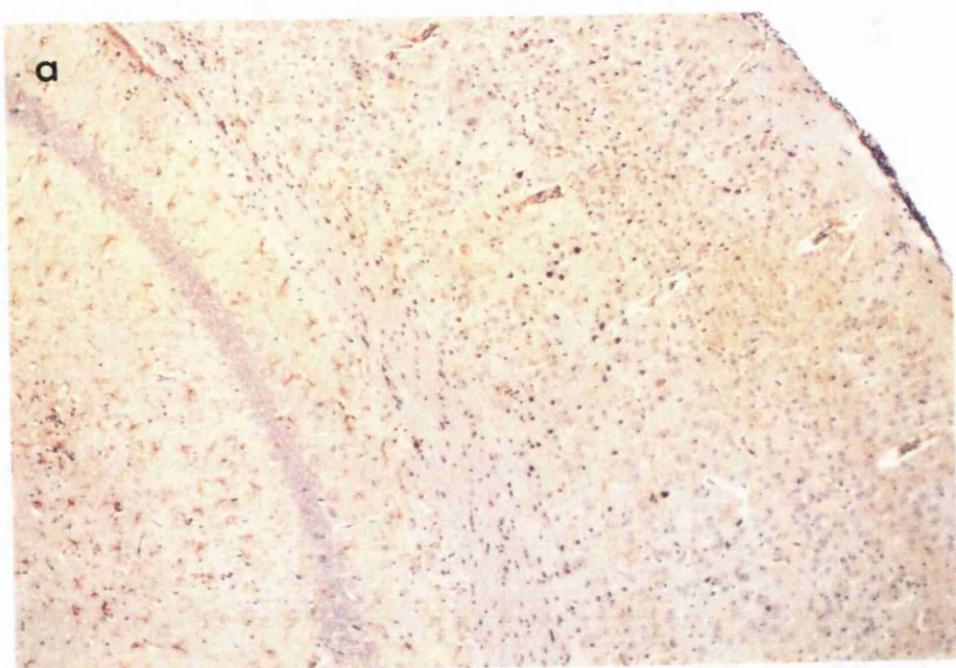
There was a slow progressive spread of astrocyte activation in *T.b. brucei*-infected, untreated mice. Thus, astrocyte activation first seen in the corpus callosum, along the meninges and the choroid fissure, spread outwards into the brain parenchyma, first the white and then the grey matter. By day 52 after infection, astrocyte activation had spread to form a wide band around the corpus callosum, involving the adjacent grey matter. Few activated astrocytes were scattered in the rest of the grey matter, with more of these astrocytes concentrated around the blood vessels (Figure 3.3a).

Mononuclear inflammatory cells, including lymphocytes, plasma cells and macrophages, were first observed in the choroid fissure and the meninges in mice sacrificed 28 days after infection. At this time, infiltration in both the meninges and the choroid fissure, was focal and only 1 or 2-cells thick. Astrocyte activation was confined to around the choroid fissure, along the meninges, in the hippocampus and the corpus callosum (Figures 3.2b and d), and around the large blood vessels in the cerebellum (Figure 3.2f). As the disease progressed, the mononuclear cell infiltrations increased to form a continuous layer in the meninges and the choroid fissure. By day 52 after infection, there was a continuous layer of inflammatory cells in the entire circumference of the meninges and in the choroid fissure, 5 or more cells thick. In addition perivascular cuffs were evident in the hippocampus and the corpus callosum (Figures 3.3a and b). As the inflammatory cell infiltrate increased so did the spread of activated astrocytes into the parenchyma.

Similarly, mononuclear inflammatory cells were found infiltrating the cerebellar meninges, folds and the perivascular spaces at the base and the lobes, 28 days after infection. At this time, the infiltrates were only 1 or 2-cells thick and astrocyte activation was confined to the white matter and around the blood vessels in the granular layer (Figure 3.2f; Figure 3.4a). As in the cerebrum, the infiltrates increased, thickening the meninges and enlarging the perivascular

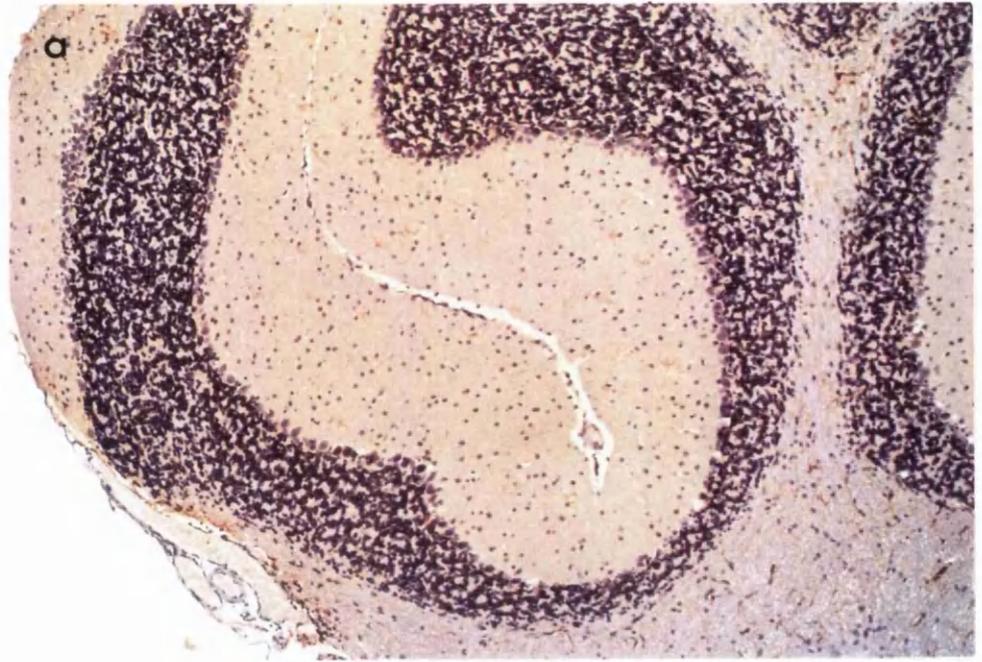
### Figure 3.3

Brain sections from mice killed after 52 days of infection with *T.b. brucei* (group IC). The number, size, degree of stellation and the GFAP staining intensity of astrocytes in the corpus callosum (a) and the hippocampus (b) are increased. A few activated astrocytes can be found scattered in the cerebral grey matter with the majority of them occurring around the blood vessels (a). Note that the distribution of activated astrocytes in the cerebral grey matter is not diffuse at this time. There is moderate infiltration of the meninges (a) and the choroid fissure (b) by mononuclear inflammatory cells. The sections were immunostained with rabbit anti-GFAP (DAKO), developed with DAB/peroxidase kit (Vectastain) and counterstained with haematoxylin. x90



### Figure 3.4

Cerebellar sections from mice infected with *T.b. brucei* (group IC) and sacrificed on day 28 (a) and day 52 (b) after infection. Note that after 28 days of infection, activated astrocytes are confined to the base of the cerebellum (a) and how astrocyte activation spreads to the cerebellar lobes (b). Some activated astrocytes can be seen in the granular layer of the cerebellum 52 days after infection (arrows). The sections were stained with rabbit anti-GFAP antibody, developed with DAB/peroxidase kit and counterstained with haematoxylin. x90



cuffs. By day 52 after infection, there was a 5 to 10-cell layer of mononuclear inflammatory cells in the meninges and the meningeal folds and prominent perivascular cuffs in the white matter of the cerebellar lobes (Figure 3.4b).

There was no difference observed between the pathology caused by the two trypanosome stabilates. Uninfected mice treated with Berenil did not develop any CNS pathology.

### **3.3.2 Astrocyte activation in post treatment reactive encephalitis**

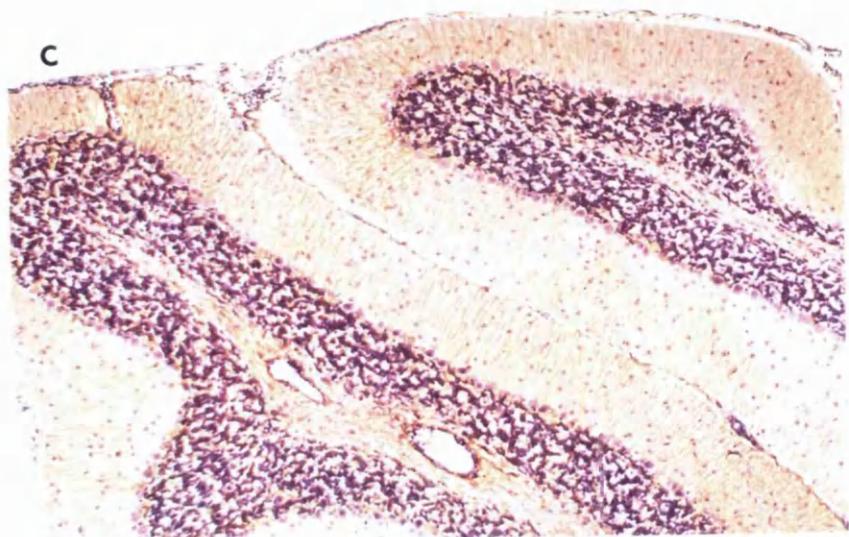
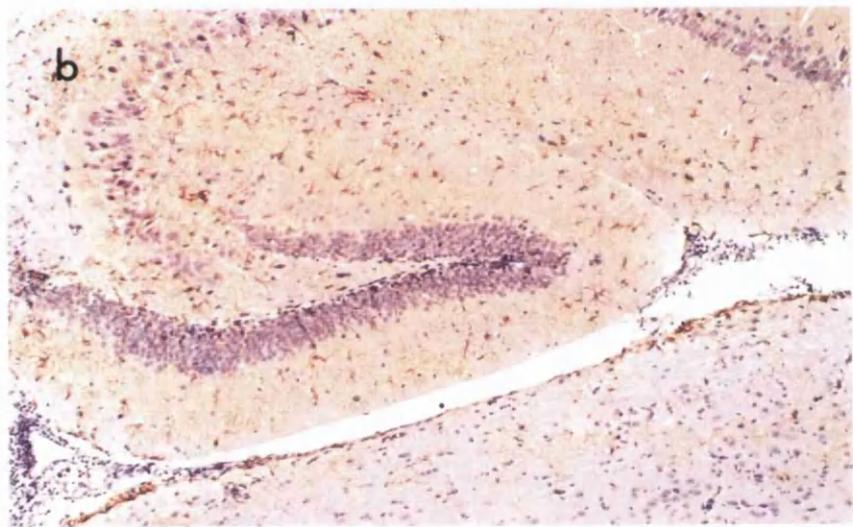
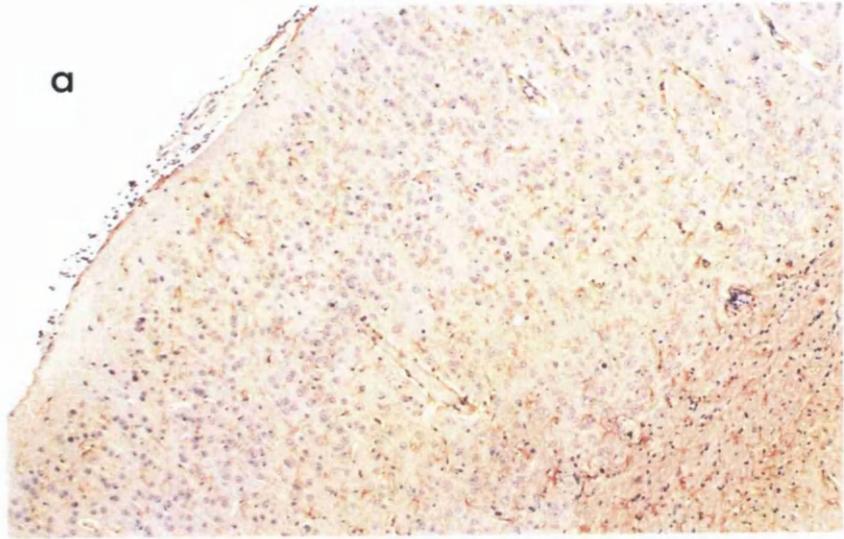
Brain sections of mice treated with Berenil on day 21 after infection and sacrificed 7 days later (day 28 after infection), showed active astrocytes diffusely distributed in the brain parenchyma in both the white and the grey matter of the cerebral cortex (Figure 3.5a). Astrocytes in the hippocampus were more in number, stained more intensely for GFAP and were more elaborately stellate than those in the grey matter (Figure 3.5b). In the cerebellum, activated astrocytes were found throughout the white matter (Figure 3.5c). In addition, the meninges, the choroid fissure and the perivascular spaces were infiltrated by mononuclear inflammatory cells, including, lymphocytes, plasma cells and macrophages. Perivascular cuffs were also present in the white matter at the base and in the cerebellar lobes (Figures 3.5c).

The spread of astrocyte activation in this group of mice occurred faster than in the infected, untreated mice. Activated astrocytes were diffusely distributed throughout the cerebral and the cerebellar cortices by day 43 after infection (22 days after induction of PTRE with Berenil) (Figures 3.6a and b). At this time, there were large perivascular cuffs in the cerebral white and grey matter, the cerebellar white matter and numerous inflammatory cells in the meninges and meningeal folds. Astrocytes around the inflammatory-cell foci, appeared more activated than those in the rest of the parenchyma (Figure 3.6a). In the cerebellum, activated astrocytes were found in the white matter and in both the molecular and the granular layers of the grey matter. In addition, the

### Figure 3.5

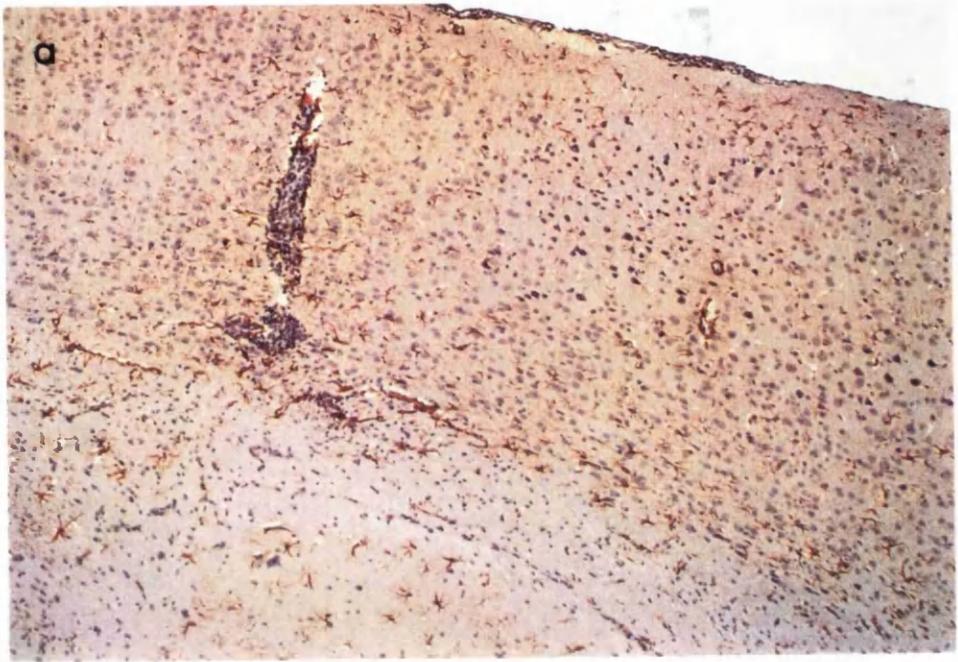
Brain sections of a mouse from group P1, that was infected with *T.b. brucei*, treated with Berenil 21 days later and sacrificed on day 28 after infection, i.e., 7 days after Berenil injection. Note that the meningitis in (a) is greater than in untreated mice sacrificed 52 days after infection (See Figure 3.4). There are activated astrocytes in the cerebral (b) and the cerebellar white matter (c). Numerous inflammatory cells can be seen infiltrating the choroid plexus (b) and there are perivascular cuffs in the cerebellar white matter (c). The sections were immunostained with rabbit anti-GFAP (DAKO), developed with DAB/peroxidase kit (Vectastain) and counterstained with haematoxylin.

x90



### Figure 3.6

Diffuse distribution of activated astrocytes in the cerebral (a) and cerebellar (b) cortices of a mouse treated with Berenil on day 21 and sacrificed on day 43 after infection (group P1). Note that astrocytes appear more active around perivascular cuffs. Bergman glia in the molecular layer, of the cerebellum, stain for GFAP (arrows). The sections were immunostained with rabbit anti-GFAP (DAKO), developed with DAB/peroxidase kit (Vectastain) and counterstained with haematoxylin. x90



Bergman glia in the molecular layer stained for GFAP (Figure 3.6b).

Mice that received a second Berenil treatment after the relapse of parasitaemia showed an exacerbation of this CNS reaction. Thus, 7 days after the second Berenil treatment, there was an apparent increase in the number and the degree of astrocyte activation, as judged by morphological appearance and the intensity of GFAP staining. Activated astrocytes were distributed throughout the brain parenchyma. There was also an increase in the size of perivascular cuffs, and the number of mononuclear inflammatory cells infiltrating the meninges and the choroid fissure (Figures 3.7a to d). At this time, the astrocytes were enlarged, had many (four or more), long, elaborately branched cytoplasmic processes and stained very intensely for GFAP (Figure 3.8a). Serial haematoxylin and eosin-stained sections showed the presence of trypanosomes in the brain parenchyma (Figure 3.8b).

Infected, treated mice showed relapse of parasitaemia 20-22 days after treatment with Berenil. The pathological changes were identical in mice that were infected with the eflornithine-sensitive and the eflornithine-resistant trypanosome stabilates. Uninfected mice treated with Berenil once or twice did not show any pathological lesions.

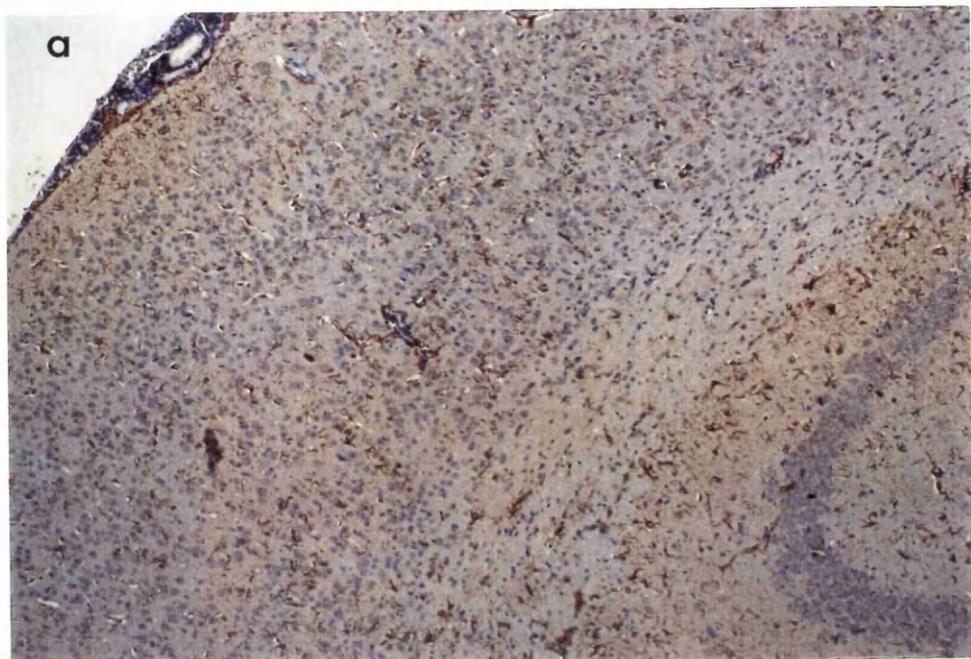
### **3.3.3 Astrocyte activation after curative trypanocidal therapy**

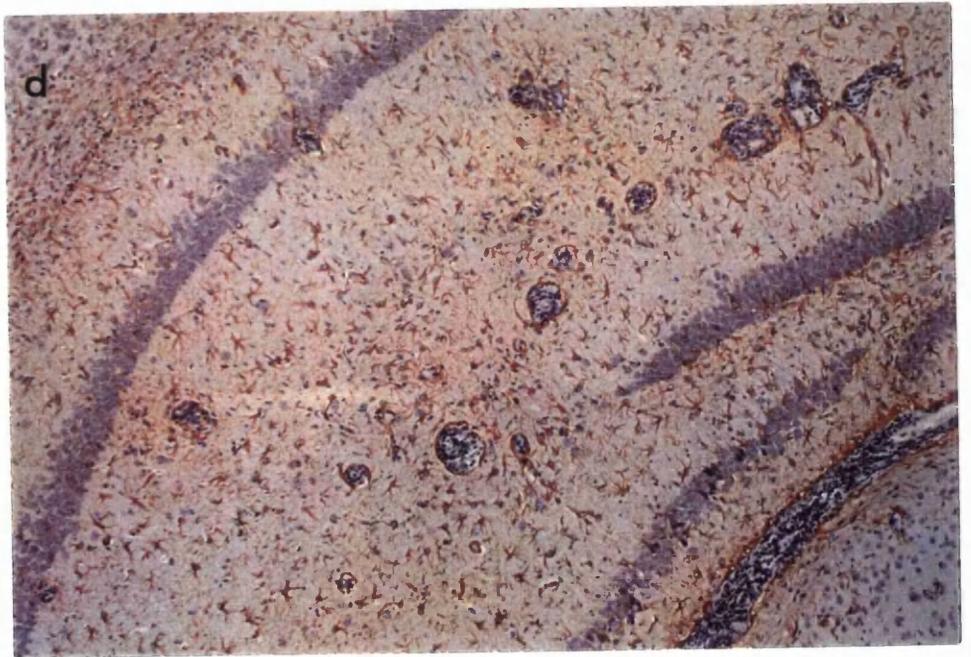
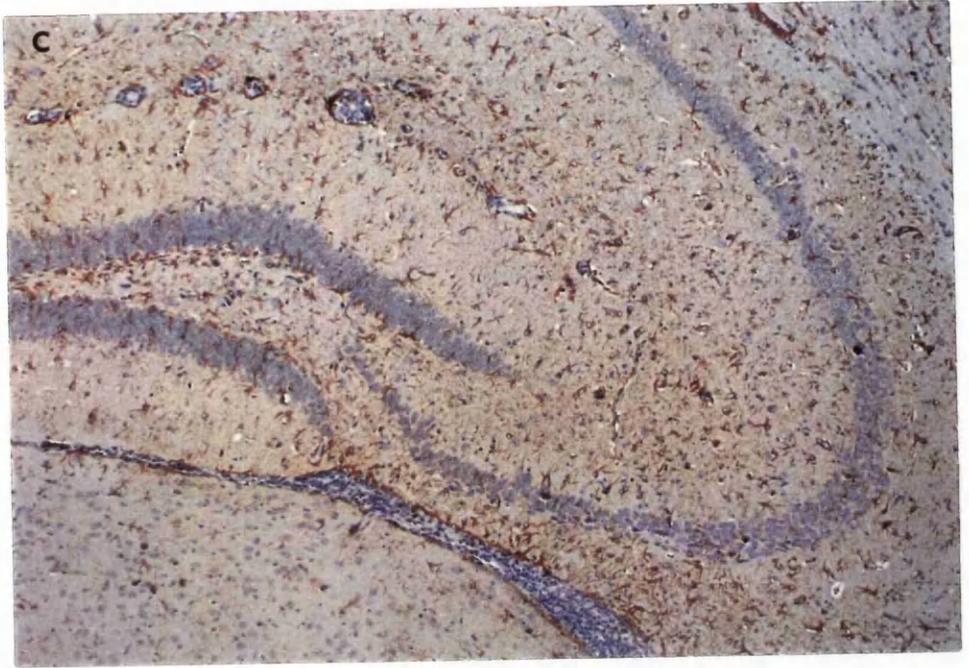
Curative trypanocidal therapy after a double-Berenil regimen, was followed by a progressive resolution of the CNS pathology. The disappearance of inflammatory cell infiltrations preceded the resolution of astrocyte activation.

In mice killed 5 days after curative treatment, there was a remarkable decrease in inflammatory-cell infiltration, in the meninges and the perivascular cuffs. Complete resolution of the meningitis was evident 12 days after curative treatment. However, encephalitis characterised by a few inflammatory cells in the perivascular spaces within the hippocampus and in the choroid fissure, was

### Figure 3.7

Comparison of the degree of astrocyte activation and inflammation in the brain of a mouse from group P1, that received a single Berenil treatment on day 21 and killed on day 52 after infection (a and c), to the brain of a mouse from group P2, that received Berenil on day 21 after infection and again after relapse of parasitaemia (day 45 after infection), then sacrificed 7 days (day 52 after infection) after the second Berenil (b and d). There is an increase in the number of astrocytes, the degree of astrocyte stellation and the intensity of GFAP-stain in mice that received the double-Berenil regimen (b and d). Note also the increase in the numbers of mononuclear inflammatory cells infiltrating the meninges (b), the perivascular cuffs (b and d), and the choroid fissure (d). The sections were immunostained with rabbit anti-GFAP (DAKO), developed with DAB/peroxidase kit (Vectastain) and counterstained with haematoxylin. Magnification. x90

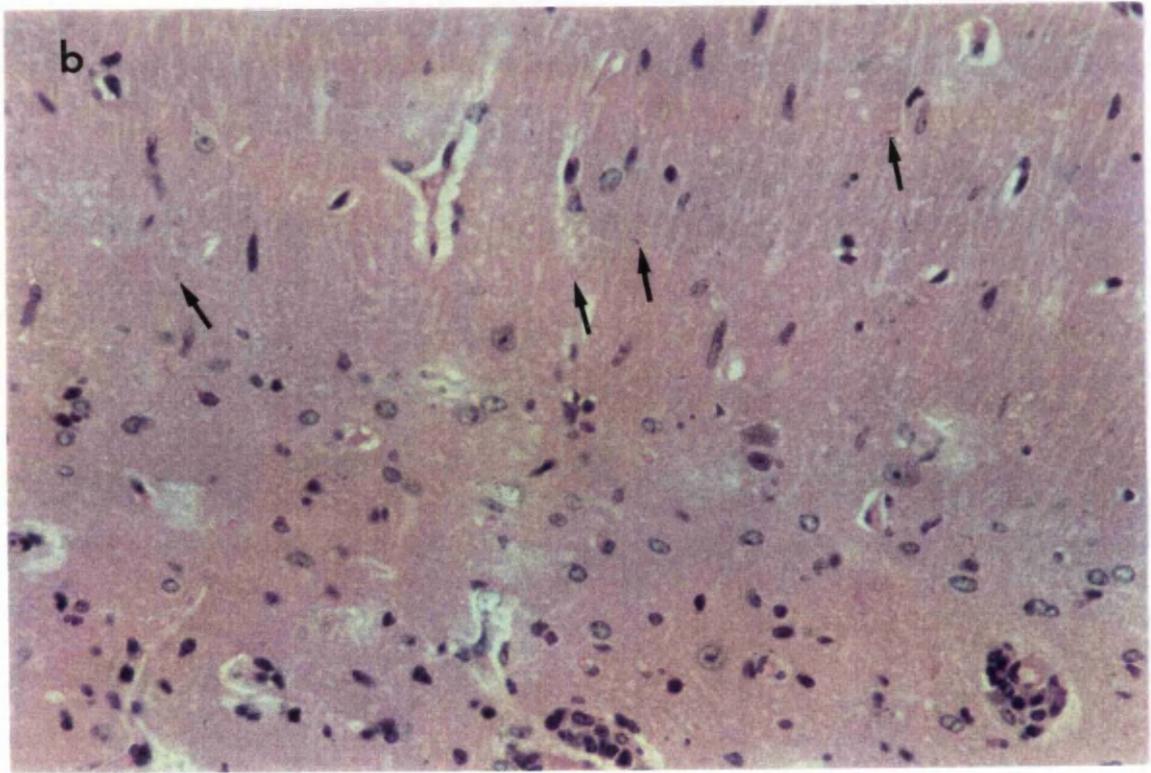
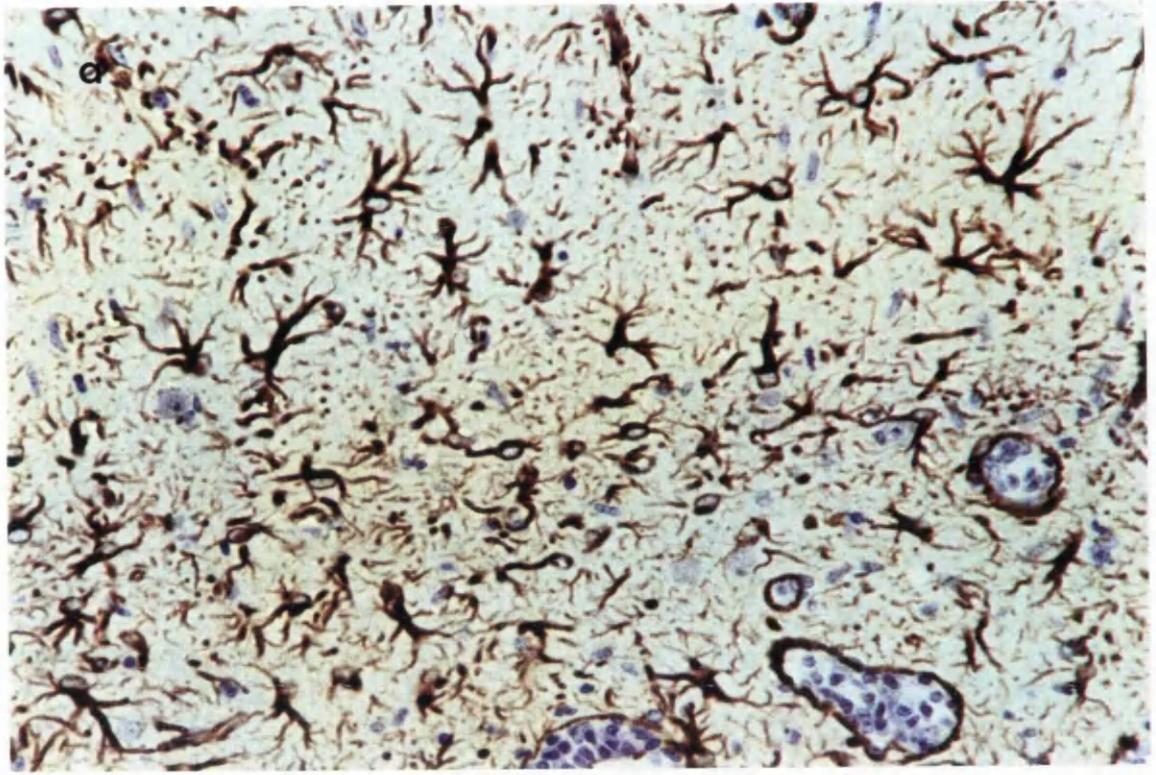




### Figure 3.8

The cerebrum of a mouse from group P2, treated with Berenil on day 28 after infection and again after relapse and killed 14 days after the second Berenil treatment. Figure 3.8a shows the large elaborately stellate astrocytes diffusely distributed in the brain parenchyma. The cytoplasmic processes are multiple and branched and stain intensely for GFAP. This section was stained with rabbit anti-GFAP antibody, developed with DAB/peroxidase kit (Vectastain) and counterstained with haematoxylin. Figure 3.8b shows a serial section of the same area, from the same mouse, stained with haematoxylin and eosin. Note the trypanosomes in the parenchyma (arrows) not associated with perivascular cuffs.

x360



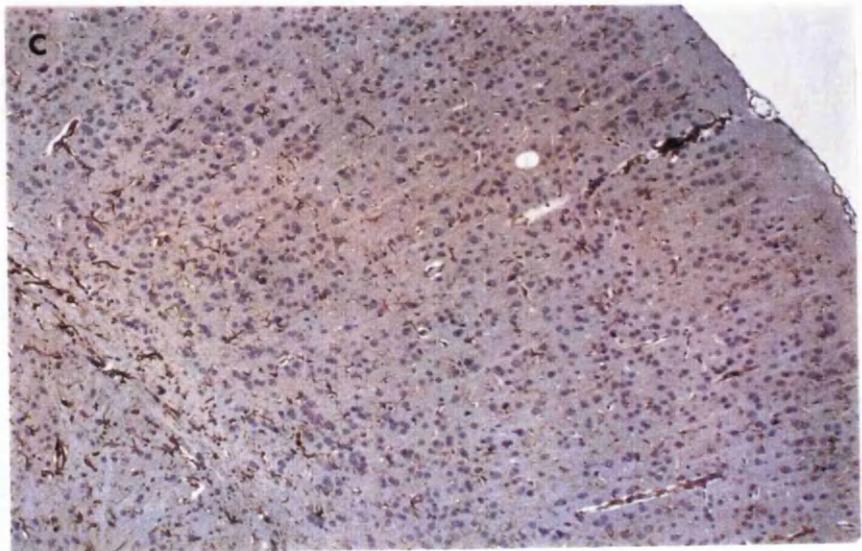
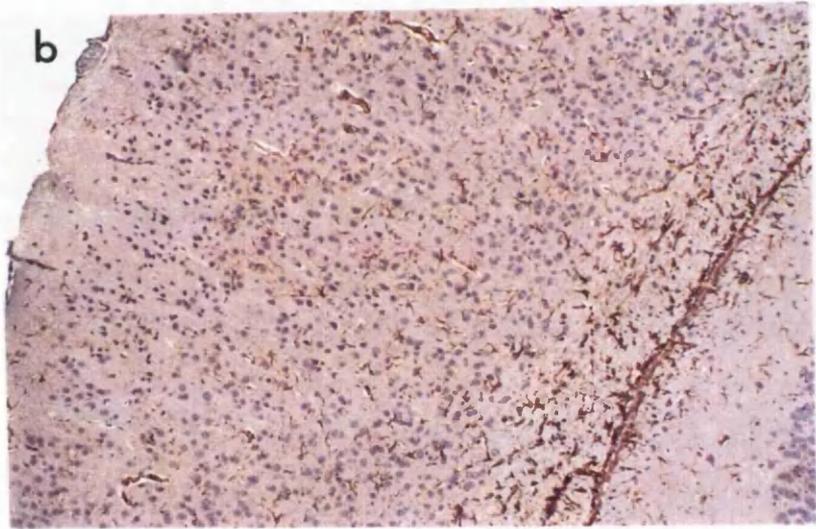
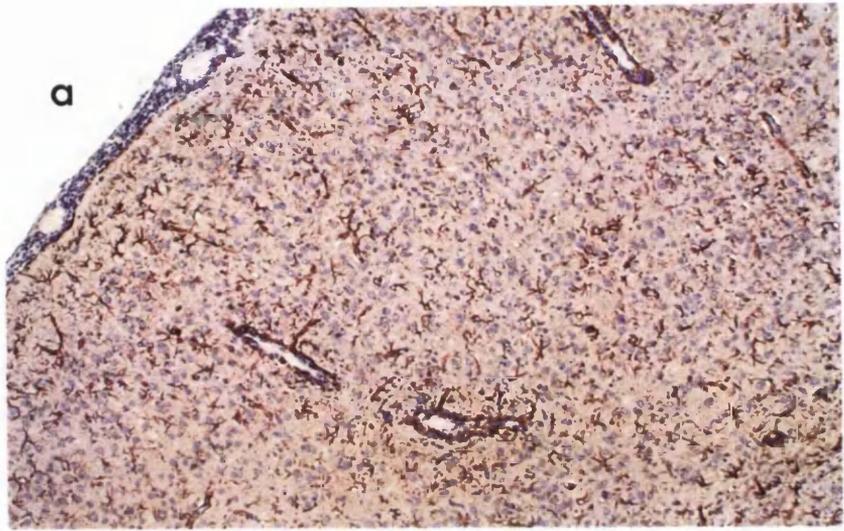
still present. By day 26 after curative treatment, focal, small accumulations of inflammatory cells could be found only in the choroid fissure. The disappearance in the inflammatory cell infiltrations occurred faster in the cerebellum than in the cerebrum. Thus, the cerebellum showed a remarkable reduction in the meningeal cellular infiltrates 5 days after curative treatment. Twelve days after curative treatment, the meningeal infiltrates had disappeared leaving only a few inflammatory cells remaining in the perivascular spaces at the base of the cerebellum. By day 19 after curative therapy, the inflammatory cell infiltrations in the cerebellum had disappeared completely.

The resolution in astrocyte activation was slower and appeared to be graduated, disappearing faster in the grey matter and taking longer in the white matter. Thus, there was a reduction in the GFAP-staining of activated astrocytes in the cerebral grey matter 5 and 12 days after curative therapy. However, at this time point, astrocytes in the hippocampus were still comparable to those in sections from mice sacrificed before curative therapy (Figures 3.9a and 3.10a). By day 19 after curative trypanocidal therapy, astrocyte activation was moderate in the cerebral grey matter and moderately severe in both the corpus callosum and the hippocampus although most of the inflammatory cells had disappeared (Figures 3.9b and 3.10b).

However, this steadily decreased so that 26 days after curative treatment, the resolution of astrocyte activation in the cerebral grey matter was almost complete with only a few, poorly stellate, faintly GFAP-staining astrocytes remaining (Figure 3.9c). Although moderate astrocyte activation was still evident in the hippocampus, the size, the intensity of GFAP staining and the degree of stellation of these astrocytes was reduced when compared to the mice sacrificed 19 days after curative therapy and the Berenil treated controls (Figures 3.10c).

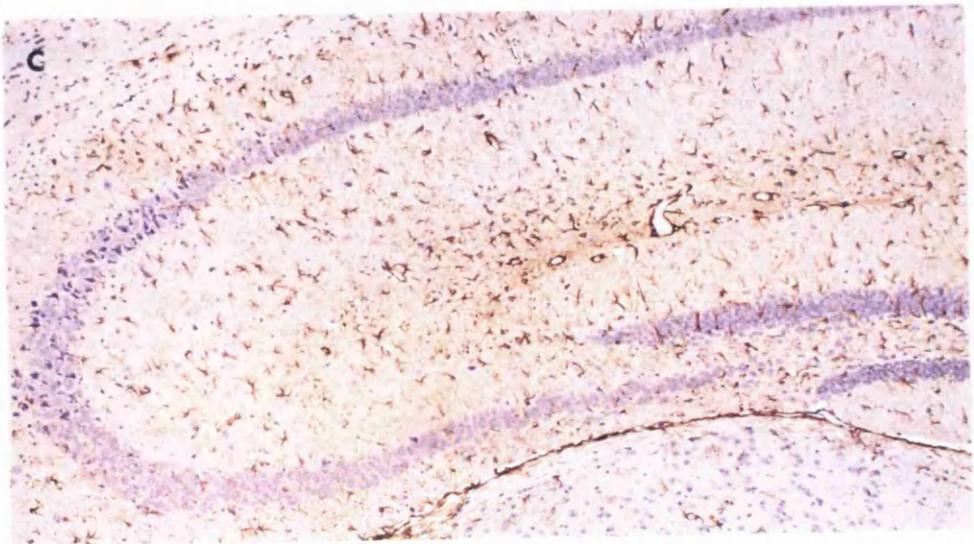
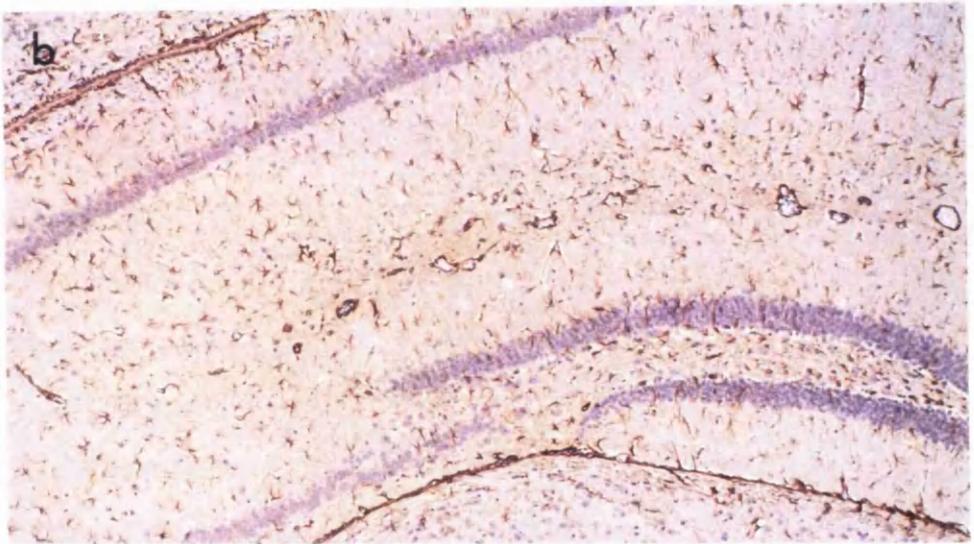
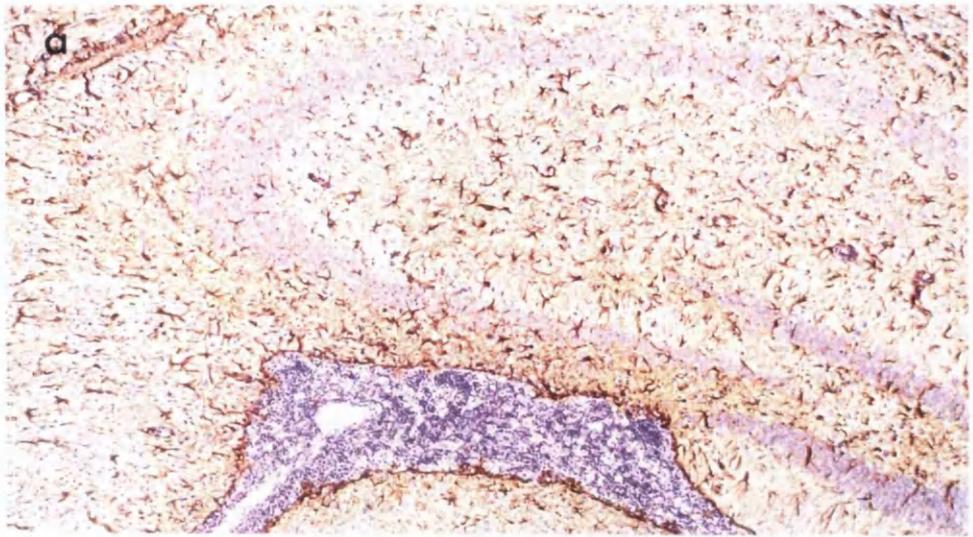
### Figure 3.9

Comparison of astrocyte activation in the cerebral grey matter of a mouse from group P2, treated with the double-Berenil regimen and killed 12 days later (a), with that of mice, from group CT, that were treated with the curative regimen of 15mg/kg MK 436 and 5mg/kg Mel Cy for 2 consecutive days, starting on the 7<sup>th</sup> day after the second Berenil treatment, and sacrificed 19 (b) and 26 (c) days later. Note how the activated astrocytes disappear from the grey matter first (b) and then from the corpus callosum (c). Note also the reduction in the size, degree of stellation and intensity of GFAP staining in curatively treated mice (b and c). Astrocyte activation in the grey matter is almost completely resolved 26 days after curative therapy (c). The sections were immunostained with rabbit anti-GFAP (DAKO), developed with DAB/oxidase kit (Vectastain) and counterstained with haematoxylin. x90



### Figure 3.10

Comparison of astrocyte activation in the hippocampus of a mouse from group P2, treated with the double-Berenil regimen and killed 12 days later (a), with that of mice, from group CT, that were treated with the curative regimen of 15mg/kg MK 436 and 5mg/kg Mel Cy for 2 consecutive days, starting on the 7<sup>th</sup> day after the second Berenil treatment, and sacrificed 19 (b) and 26 (c) days later. Note how the astrocytes decrease in size and in the degree of stellation. Note also how the perivascular cuffs have gradually disappeared (b) and (c). By 26 days after curative therapy, inflammatory cells have disappeared from the perivascular spaces and the choroid fissure (c). The sections were stained with rabbit anti-GFAP, developed with DAB/peroxidase kit and counterstained with haematoxylin. x90



Astrocyte activation 5 and 12 days after curative treatment was comparable to that seen in the double-Berenil-treated controls (Figure 3.11a). Nineteen days after curative therapy, there was a decrease in the intensity of GFAP-stain in the activated astrocytes within the white matter and the Bergman glia in the molecular layer of the cerebellum (Figure 3.11b). This decreased further so that by day 26, activated astrocytes were confined to the white matter at the base of the cerebellum and there was only a faint GFAP-stain on the Bergman glia (Figure 3.11c).

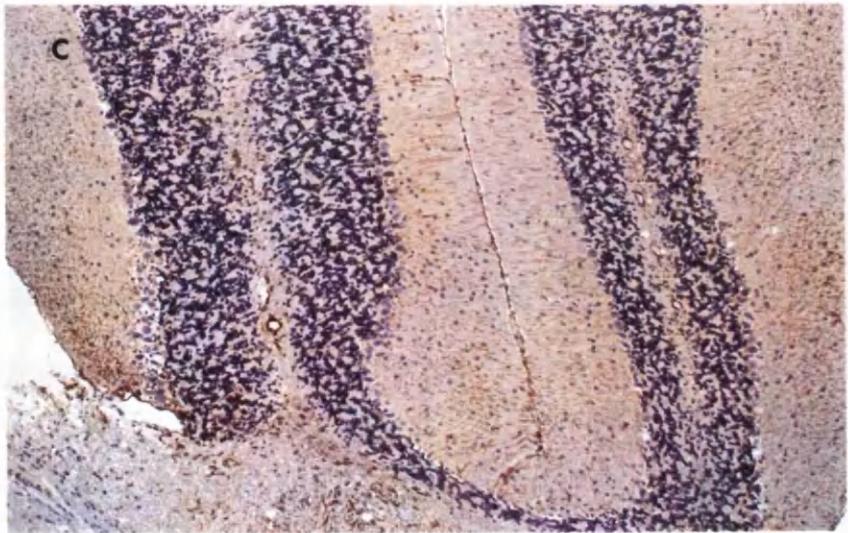
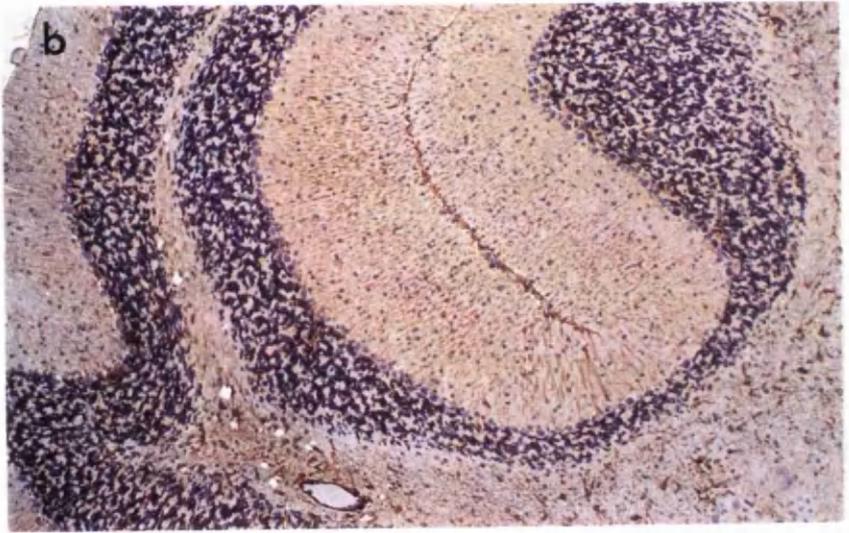
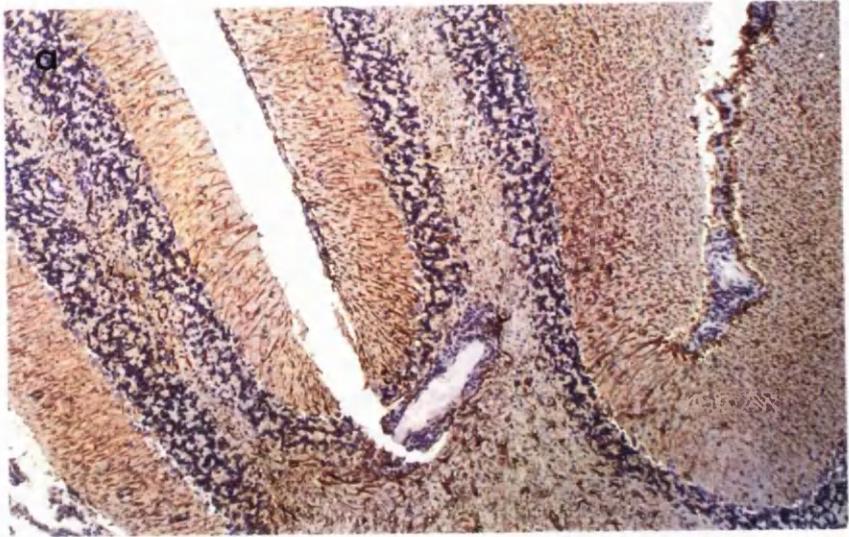
The response to curative therapy was the same whether the infecting trypanosome stabilate was eflornithine-sensitive or eflornithine-resistant. Uninfected mice treated with this combination of drugs did not show any pathological lesions in the CNS.

### 3.4 DISCUSSION

These results confirm the observation that astrocyte activation in this mouse model, is the first pathological sign of neurological involvement after infection with African trypanosomes and that it occurs before the infiltration of the CNS by inflammatory cells (Hunter *et al*, 1992b). Astrocyte activation was observed 21 days after infection. Astrocyte activation, in this study, was judged by the increase in staining of the glial fibrillary acidic protein (GFAP), the predominant protein in the astrocyte intermediate filaments (Eng, 1985), and by the morphological changes, i.e., the increase in size and the degree of stellation. Astrocyte activation occurred first around the ventricles, along the choroid fissure, along the pineal recess, in the pineal gland, in the hippocampus and in the corpus callosum. As these are areas where the blood-brain barrier is incomplete, the activation might be the response of astrocytes to trypanosome invasion. Indeed, the presence of trypanosomes in the circumventricular organs (pineal gland, area postrema and the median eminence) and the choroid plexus,

### Figure 3.11

Comparison of astrocyte activation in the cerebellum of a mouse from group P2, treated with the double-Berenil regimen and killed 12 days later (a), with that in mice, from group CT, that were treated with the curative regimen of 15mg/kg MK 436 and 5mg/kg Mel Cy for 2 consecutive days, starting on the 7<sup>th</sup> day after the second Berenil treatment, and sacrificed 19 (b) and 26 (c) days later. The disappearance of inflammatory cells from the perivascular spaces and the meningeal folds had occurred by day 19 after curative therapy (b) but the regression of astrocyte activation and the GFAP-stain in the Bergman glia was not complete after 26 days post curative treatment. However, the intensity of GFAP staining was decreased (c). The sections were stained with rabbit anti-GFAP, developed with DAB/peroxidase kit and counterstained with haematoxylin. x90



has been demonstrated to occur in mice as early as 13 days post infection (Schultzberg *et al*, 1988).

Astrocytes are one of the most numerous cell types in the CNS and they give rise to radiating processes which may extend across vast distances in the brain parenchyma to make contact with blood vessels, or with different neurones or synapses (Hertz *et al*, 1990). Due to their "strategic" placement at the blood-brain barrier, astrocytes are likely to be the first of the intrinsic CNS cells to come into contact with the trypanosome signal emanating from the systemic blood circulation. This may explain why astrocyte activation appears to occur before the infiltration of inflammatory cells into the meninges and the brain parenchyma.

Activated astrocytes are phagocytic, produce cytokines and interact with antigen-specific T cells to control immune reactivity within the CNS (Pentreath, 1989; Wekerle *et al*, 1986; Hertz *et al*, 1990) for instance, by presenting antigens to MHC-restricted T cells (Vidovic, Sparacio, Elovitz and Benveniste, 1990; Sedgwick, Mobner, Schwender and ter Meulen, 1991). Previous work has shown that astrocytes can be induced to produce various cytokines and growth factors notably IL-1 $\alpha$ , IL-6 and TNF $\alpha$  (Saukkonen *et al*, 1990; Lieberman *et al*, 1989), glial cell stimulating factor (GSF) (Fontana, Otz, de Weck and Grob, 1981) and glial promoting factor (GPF) (Giulian and Baker, 1985). It is possible that astrocytes produce such cytokines during trypanosome infection and initiate the meningeal inflammation seen in African trypanosomiasis. Indeed, cytokine transcripts for IL-1, IL-4, TNF $\alpha$ , IFN $\gamma$ , and MIP-1 have been shown to occur, and to closely correlate with the onset of astrocyte activation in the CNS of *T.b. brucei*-infected mice (Hunter *et al*, 1991).

The cytokines, IL-1 $\alpha$  and TNF $\alpha$  can induce: proliferation, expression of MHC class II antigens and adhesion molecules, and production of cytokines by

various cells. The affected cells include, astrocytes themselves (McCarron, Spatz, Kempinski, Hogan, Muehl and McFarlin, 1986), macrophages (Rouzer and Cerami, 1980), endothelial cells (Montovani and Dejana, 1989), microglia (Ramilo *et al*, 1990; Saukkonen *et al*, 1990; Hurwitz, Lyman, Guida, Calderon and Berman, 1992; Lee, Liu, Roth, Dickson, Berman and Brosnan, 1993b) and lymphocytes (Selmaj *et al*, 1990). All these cells are found in the CNS during late-stage trypanosome infections in the mouse (Poltera *et al*, 1980a; Jennings *et al*, 1989; Hunter *et al*, 1991), and man (Ormerod, 1970; Poltera, 1980; Haller *et al*, 1986; Adams *et al*, 1986). Expression of adhesion molecules on endothelial cells would cause extravasation of leukocytes into the perivascular spaces (Montovani and Dejana, 1989), while expression of these molecules on astrocytes and microglia would facilitate the spread of infiltrating cells into the CNS parenchyma (Hurwitz *et al*, 1992). Proliferation of the inflammatory cells would enlarge the perivascular cuffs while proliferation of the microglia and astrocytes would cause astroglial hyperplasia and activation, all of which are major features of late stage trypanosomiasis.

It is also possible that the cytokines produced act in an autocrine and paracrine manner to induce further astrocyte activation and cause leukocyte extravasation and proliferation, producing the characteristic progressive CNS lesion seen in African trypanosomiasis. For instance, astrocytes have been shown to respond to IL-1 and TNF $\alpha$  by producing macrophage chemotactic and activating factors (Kasahara, Mukaida, Yamashita, Yagisawa, Akahoshi and Matsushima, 1991), colony stimulating factor 1 (Thery, Hetier, Evrard and Mallat, 1990), IL-3 and GM-CSF (Fontana *et al*, 1981; Merrill, 1991); and IL-1, IL-6 and TNF $\alpha$  (Lee, Liu, Dickson, Berman and Brosnan, 1993a), all of which act in co-operation to effect leukocyte extravasation and proliferation, essentially making perivascular cuffs.

The finding in the CNS of normal mice, studied here, that astrocytes around the ventricles and in the circumventricular organs stain for GFAP, albeit

faintly, suggests a pre-activated status of astrocytes in these areas, possibly as a result of the incomplete blood-brain barrier in these regions. It seems as if these astrocytes keep "armed guard" against intrusion by invading pathogens. During trypanosome infections these astrocytes become fully activated (hence the increase in the intensity of GFAP staining) to deal with the invading trypanosomes before they infiltrate the CNS parenchyma. Since activated astrocytes are phagocytic, produce cytokines and present antigens (Pentreath, 1989; Wekerle *et al*, 1986; Hertz *et al*, 1990; Vidovic *et al*, 1990), it is possible that during trypanosome infections, the activated astrocytes phagocytose trypanosomes, produce leukocyte-chemotactic cytokines and present trypanosome antigen to the infiltrating lymphocytes. It would appear that the initial astrocyte activation could be an attempt by this cell type to prevent invasion of the brain parenchyma by trypanosomes. This would also focus inflammatory reactions at the edge of the CNS parenchyma, where immediate drainage of the resultant inflammatory products into the blood circulation might occur, avoiding any destructive effects of these products on the brain parenchyma. Furthermore, the production of CSF by the choroid plexus within the ventricles might facilitate this drainage. However, constant stimulation by an unresolved trypanosome infection is likely to lead to increased astrocyte activation and cytokine production, finally leading to the progressive CNS lesion found in late-stage trypanosomiasis.

The results in this study also show that in the infected, untreated mice, inflammatory cell infiltration and astrocyte activation spread slowly: even by day 52 after infection, astrocyte activation was not completely diffuse, with most of the cerebral grey matter free of activated astrocytes. In addition, the inflammatory cell infiltration was restricted to the meninges and the choroid fissure with only a few scattered perivascular cuffs in the white matter. This is not surprising considering the immune privileged status of the central nervous

system. For instance, the normal CNS produces mediators such as the neurotransmitters, glutamate and norepinephrine, that downregulate the IFN $\gamma$ -mediated MHC class II antigen expression on astrocytes (Frohman, Frohman, Dustin, Vayuvegula, Choi, Gupta, Van der Noort and Gupta, 1989; Lee, Liu, Brosnan and Dickson, 1992). In addition, the interface between the blood and the central nervous tissue consists of concentric layers of endothelial cells, basal membranes and astrocyte endfeet, which constitute a physical blood-brain barrier to molecules even as small as monovalent ions (Hertz *et al*, 1990). Astrocytes themselves, constitutively express the gene for colony stimulating factor 1 (CSF-1), a cytokine that downregulates the IFN $\gamma$ -induced expression of MHC class II antigens on microglial cells (Lee *et al*, 1993b). This would reduce antigen presentation by astrocytes and microglia, curbing inflammatory reactions within the CNS.

This study further shows that astrocyte activation increases as inflammatory cells infiltrate the meninges, and becomes diffusely distributed in the parenchyma as the severity of meningoencephalitis progresses. This suggests that although the initial astrocyte activation in this infection is not dependent on inflammatory cells, the latter may be playing an important part in boosting the activation and the spread of activated astrocytes that occur in the CNS during chronic trypanosome infections. The observation in this study that astrocytes appear more activated around perivascular cuffs supports this suggestion. Other investigators (Yong *et al*, 1991) found that IFN $\gamma$ , a product of activated lymphocytes, enhances reactive gliosis in adult mice and induces proliferation of human astrocytes *in vitro*. Since the infiltrating cells in trypanosome infections are mainly lymphocytes (Schultzberg *et al*, 1988), it is possible that IFN $\gamma$  is produced and enhances astrocyte activation. Indeed, IFN $\gamma$  is one of the cytokines reported to be produced in the CNS of *T.b.brucei*-infected mice (Hunter *et al*, 1991). The cells that infiltrate the perivascular spaces and later the brain parenchyma are themselves capable of producing cytokines when

stimulated with various stimuli including lipopolysaccharide and cytokines (Yong *et al*, 1991; Olsson, Bakheit, Edlund, Hojeberg, Van der Meide and Kristensson, 1991; Merrill, Kono, Clayton, Ando, Hinton and Hofman, 1992); in addition to being target cells for these cytokines. It would appear that astrocytes and inflammatory cells work together to aggravate the inflammatory lesion in the CNS during trypanosome infections.

This study also confirms the findings by other workers (Jennings and Gray, 1983; Jennings *et al*, 1989; Hunter *et al*, 1992c) that treatment with Berenil, amongst other drugs (Jennings and Gray, 1983), enhances both the inflammatory cell infiltration and astrocyte activation. It has been suggested that the rapid disappearance of all the trypanosomes from other parts of the body allows the immune system, which up to this time was "swamped" by the massive numbers trypanosomes in the circulation, to concentrate on the surviving parasites in the CNS, causing meningoencephalitis (Jennings *et al*, 1989). However, relapse of parasitaemia did not lessen this reaction; instead, the CNS pathology progressively got worse.

Berenil, like other aromatic diamidines, causes release of histamines (Gall, 1954; Fussganger and Bauer, 1958; Hawking, 1963), which in turn increase vascular permeability. If the permeability of brain blood vessels is increased, there would be an influx of blood borne inflammatory cells and mediators, such as cytokines, and metabolites into the brain parenchyma. Indeed, it has been reported that Berenil increases the blood-brain barrier permeability to albumin when administered during *T.b. rhodesiense* infections in vervet monkeys (Waitumbi, Sayer and Gould, 1988; Ndung'u J.M. personal communication). Since the brain parenchyma does not provide conducive conditions for replication of trypanosomes (Bafort, Schmidt and Molyneux, 1987), blood-derived metabolites would enhance survival and replication of trypanosomes within the brain parenchyma. In addition, the blood-borne

inflammatory mediators would increase astrocyte activation as well as provide strong chemotaxis for blood-borne inflammatory cells. Both the activated astrocytes and the infiltrating inflammatory cells are capable of producing inflammatory mediators. Stimulation of these two cell types by the persisting, possibly replicating, trypanosomes in the brain parenchyma would lead to the enhanced reaction within the brain, we now know as PTRE. The observation in this study that this enhanced reaction occurs in about 7 days after treatment with Berenil, together with the report by Murray *et al* (1974b) that immunocompetence is restored in about a week after curative therapy, and the fact that Berenil clears trypanosomes in all sites except the brain parenchyma, seems to support this hypothesis. Thus, the chemotactic signal generated by the activated astrocytes and possibly the infiltrating inflammatory cells, within the brain parenchyma, is being directed at a restored immune system leading to enhanced infiltration into the brain, and enhanced reaction to the remaining trypanosomes within the brain. The observation in this study that a second Berenil treatment exacerbated the ongoing CNS pathology, and did not cure the trypanosome infection, would suggest that the disruption of the blood-brain barrier caused by Berenil is temporary, and that the continued inflammatory reaction within the CNS between parasitaemias was locally mediated, and not a mere influx of inflammatory cells and mediators from the systemic circulation. A second, possibly temporary, disruption of the blood-brain barrier might explain the exacerbation of the CNS lesion that followed this second Berenil treatment.

PTRE has been reported to occur after subcurative treatment with drugs that cross the blood-brain barrier, such as melarsoprol (Jennings *et al*, 1977; Jennings and Gray, 1983), and drugs that partially permeate the blood-brain barrier, including Berenil and suramin (de Raadt, 1966). It is possible that in crossing the blood-brain barrier, they cause temporary disruption of the blood-brain barrier, which introduces the blood-derived stimulatory boost needed for

the propagation of the CNS inflammatory process, leading to PTRE. The fact that reactive arsenical encephalopathy (RAE), in HAT, occurs after therapy with melarsoprol, as well as some early-stage drugs, seems to support this hypothesis. Trypanosome DNA has been reported to be present in brains of RAE victims (Hunter *et al*, 1992a), suggesting that RAE resulted from persisting infection. Subcurative trypanocidal therapy with drugs that partially cross the blood-brain barrier, and low doses of drugs that fully cross the blood-brain barrier, merely enhances this reaction by affecting the permeability of this barrier.

Curative trypanocidal treatment during an established PTRE resolves the astrocyte activation and the inflammatory cell infiltration. This confirms observations made by Jennings *et al* (1989) and Hunter *et al*, (1992a), and support their conclusions that PTRE results from persistent infection. The results presented here show that following curative therapy, inflammatory cells disappear faster than astrocyte activation. It would seem that with the removal of trypanosomes, astrocytes cease to produce inflammatory cytokines leading to the disappearance of inflammatory cells. Since astrocyte activation is judged by an increase in the GFAP content, the size and numbers of the cytoplasmic processes, it is possible that what is seen as persistent activation are the physical effects of previous and not of ongoing activation. This is corroborated by the observation that this apparent activation regresses with time.

The results reported in this chapter lead to the conclusion that the CNS pathological lesion is a locally mediated immune response to trypanosome invasion; and that, it is highly likely that astrocytes play a major role in initiating this reaction, possibly by producing cytokines. However, other cells of the CNS, including, microglia and neurones are also capable of producing cytokines, such as IL-1 and TNF, that would activate astrocytes. Therefore, the possibility that the astrocyte activation observed here is not the primary event

but is indirectly induced via other cells, cannot be excluded.

## **CHAPTER 4**

### **THE EFFECT OF TRYPANOSOMES, WHOLE TRYPANOSOME LYSATE AND MEMBRANE-BOUND FORM OF VARIABLE SURFACE GLYCOPROTEIN ON ASTROCYTES CULTURED *IN VITRO***

#### 4.1. INTRODUCTION

Astrocyte activation, as judged by intensity of GFAP staining, occurs prior to the infiltration of inflammatory cells into the brain of *T.b. brucei*-infected mice (Chapter 3; Hunter *et al*, 1991). This activation has been found to correlate with the induction of messenger RNA (mRNA) transcripts for the cytokines: tumour necrosis factor (TNF), interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 4 (IL-4), macrophage inflammatory protein 1 (MIP-1) and gamma interferon (IFN gamma) in the brains of infected mice (Hunter *et al*, 1991).

The cytokines, TNF and IL-1 have been reported to be important in initiating meningeal inflammatory changes in the brain (Saukkonen *et al*, 1990; Ramilo *et al*, 1990). Although cytokines produced in the CNS were originally thought to be derived from lymphocytes and macrophages, it has become obvious that cells not traditionally part of the immune system have the potential to synthesise these products. For instance, astrocytes can be induced to synthesise and secrete IL-1, IL-3, IL-6, TNF, interferon (IFN), colony stimulating factors and prostaglandin E (PGE) when stimulated with appropriate agents such as lipopolysaccharide, cytokines and neurotropic viruses (Fontana *et al*, 1982; Frei *et al*, 1985; Frei, Bodmer, Schwerdel and Fontana, 1986; Lieberman *et al*, 1986; Pentreath, 1989; Chung and Benvensite, 1990; Malipiero *et al*, 1990; They *et al*, 1992; Lee *et al*, 1993).

The early onset of astrocyte activation, and the strong correlation with the detection of several cytokine transcripts implicates astrocytes as possible initiators of the CNS inflammatory pathology that occurs in trypanosome infections. To investigate this possibility further, *in vitro* cultures of astrocytes, exposed to live trypanosomes, whole trypanosome lysate and the membrane-bound form of variable surface glycoprotein (VSGm), were tested for their ability to produce cytokines. The relationship of the cytokines detected to the

events that occur during trypanosome infections in mice are considered.

## 4.2 MATERIALS AND METHODS

### 4.2.1. Preparation of monolayers of purified type-1 astrocytes

Astrocyte preparation was performed as described by Wolswijk and Noble (1989). Dissociated cells from cerebral cortices of 2 to 3-day old NIH mice were plated at a density of  $5 \times 10^6$  in PLL-coated,  $80 \text{ cm}^2$  tissue culture flasks (Nunc). They were grown in Dulbecco's modified Eagles Medium (DMEM, Gibco) containing 1.0g/l glucose and supplemented with 10% heat-inactivated fetal calf serum (10%FCS), 2mM glutamine (Sigma) and  $25 \mu\text{g/ml}$  gentamycin (Flow Laboratories). Once a confluent monolayer of flat cells had been formed (about 7 to 10 days), the layer of process-bearing cells growing on the flat monolayer of cells was removed by treating with  $20 \mu\text{M}$ -cytosine arabinoside (AraC) (Sigma) for 48 hours to eliminate most of the faster dividing non-astrocytes. Cells were then removed from the flask by washing with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free Hanks balanced salt solution (HBSS, Gibco) followed by an incubation with 0.54mM EDTA (Sigma) in HBSS for 10 to 15 minutes, and then addition of trypsin (Bovine Pancreas type III, Sigma) to a final concentration of 300 I.U./ml. When the cells detached from the surface of the flask, the trypsinisation was stopped by adding to each flask (containing 10ml of EDTA/trypsin solution) 1ml of SBTI-DNase [5200 i.u. soybean trypsin inhibitor (Sigma) and 3.0mg/ml bovine serum albumin (BSA fraction V, Sigma) in DMEM]. Cells were triturated in a 10ml-blow out pipette, centrifuged for 10 minutes at  $89.6g$ , resuspended in HBSS. The cells were then treated in suspension with rabbit complement (Buxted Ltd, diluted 1:10), anti-A2B5 antibody (supernatant 1:1,000) (Eisenbarth *et al* 1979), the monoclonal anti-04 antibody (concentrated hybridoma supernatant 1:100) (Sommer and Schachner, 1981), and anti-galactocerebroside (GalC) monoclonal antibody (Hybridoma

supernatant 1:7) (Ranscht *et al* 1982). Treatment with these antibodies eliminated A2B5<sup>+</sup> neurones, oligodendrocytes-type 2 astrocyte progenitor cells (A2B5<sup>+</sup>, O4<sup>-</sup> /GalC<sup>-</sup>), type 2 astrocytes (A2B5<sup>+</sup>, GFAP<sup>+</sup>), and GalC<sup>+</sup> oligodendrocytes (Abney *et al*, 1983; Raff *et al* 1983a; 1983b; Sommer *et al* 1982). The remaining cells were plated onto PLL-coated flasks (Nunc) at a density of 5x10<sup>6</sup> cells/flask and maintained in 50% astrocyte conditioned medium (ACM) and 50% fresh DMEM/10%FCS (Nobel and Murray, 1984). Cultures were maintained in a humidified incubator (Flow Laboratories) at 37°C and 92.5% air and 7.2% CO<sub>2</sub> atmosphere. Purity of the cell cultures was assessed by immunofluorescence using a polyclonal rabbit anti-GFAP antibody (Raff *et al*, 1983a); 99.8% of the cells were found to have the antigenic phenotype of type-1 astrocytes (GFAP<sup>+</sup>, A2B5<sup>-</sup>).

#### **4.2.2. Trypanosomes**

Mice were irradiated with 500mCi for 10 minutes and then inoculated intraperitoneally with 10<sup>4</sup> trypanosomes prepared by dilution of a frozen stabilate. The cloned stabilate was *T.brucei*/GVR 35/1.5 which had been derived from trypanosomes isolated from a wildebeest in the Serengeti in 1966 (Serengeti/66/SVRP/10), as described in Chapter 2. When parasitaemia had reached 10<sup>9</sup> parasites/ml of blood, the mice were exsanguinated by cardiac puncture under terminal anaesthesia. Trypanosomes were separated from blood by chromatography through a DEAE cellulose column as described by Lanham (1968) as outlined in Chapter 2. Trypanosomes were used at a concentration of 10<sup>4</sup> and 10<sup>7</sup> parasites/ml of medium.

#### **4.2.3. Trypanosome lysate**

Whole trypanosome lysate was prepared by freeze thawing a pellet of trypanosomes by placing in liquid nitrogen, removing and allowing it to thaw at room temperature. The pellet was then subjected to 2 hours of sonication in a

bath sonicator at 4°C. The concentration of the lysate was estimated by measuring the protein content using the Biorad method of protein assay (Bradford, 1976). Trypanosome lysate was used at two different concentrations: 25µg/ml and 50µg/ml. These concentrations were chosen because, 25µg/ml VSG has been reported to stimulate cytokine production in cultured macrophages (Mathias, Perez and Difley, 1990).

#### **4.2.4. Purification of membrane-bound form of variable surface glycoprotein**

Purification of the membrane-bound form of variable surface glycoprotein (VSGm) was performed as described by Jackson *et al* (1985). Briefly, a suspension of bloodstream forms of *T.b.brucei* (about  $1 \times 10^{10}$  cells) in 20ml of Krebs-Ringers phosphate buffer (Voorheis, 1980) was treated at 0°C with an equal volume of 10% w/v trichloroacetic acid (to inactivate the coat-releasing enzyme) and the precipitate was sedimented by centrifugation at 9000g for 10 seconds. It was critical to obtain a loose pellet at this stage. The material in the pellet was resuspended in distilled deionised water ( $4 \text{ml}/10^{10}$  cells) with the aid of a 1ml pipette. The suspension was extracted with 20 volumes of chloroform/methanol (2:1, v/v) by vigorous shaking for 5 minutes followed by storage overnight at 4°C. This storage procedure has been found to increase the final yield of purified VSG (Jackson *et al*, 1985). The extract was then separated into 2 phases by the addition of 0.2 volume of 0.9% sodium chloride solution, and was centrifuged at 12,000g for 1 hour. The upper (aqueous) phase contained pure VSGm, and was removed by aspiration and dialysed against 60 litres of distilled deionised water at 4°C, with 15 changes of water, over a duration of 36 hours (Chapter 2). The retained material was freeze-dried and the resulting VSG stored dry over  $\text{P}_2\text{O}_5$  *in vacuo* until required. This method recovers over 90% of VSGm in the organic solvent (Jackson *et al*, 1985).

VSGm is partially soluble in water but dissolves completely if sufficient 1M NaOH is added to bring the suspension to pH 10.0 followed immediately by neutralisation with 1M Tris HCl (pH 7.0). A separate titration was performed to determine amount of Tris required for neutralising 1M NaOH. The freeze dried VSGm was weighed and dissolved as described above, to make a stock solution. This stock was diluted appropriately with the culture medium to a final concentration of 25µg/ml and 50µg/ml solutions for adding to astrocyte cultures.

#### **4.2.5. Lipopolysaccharide**

The lipopolysaccharide (LPS) used in this study was purchased from Sigma Laboratories and was a chromatographically purified phenol extract derived from *Escherichia coli* serotype 026:B6. The concentration used in this assay was 10µg/ml. At this concentration, LPS has been reported to stimulate astrocytes, optimally, to produce cytokines (Lieberman *et al*, 1989) and was used to compare with the various stimuli tested in this study.

#### **4.2.6. Stimulation of astrocytes**

The cells were divided into seven groups namely E, T1, T2, L1, L2, V1 and V2 (Table 4.1). The culture medium was removed and replaced with one that contained the test reagents. Thus, group E was exposed to LPS at 10µg/ml; groups T1 and T2 were stimulated with live trypanosomes at 10<sup>4</sup> and 10<sup>7</sup> trypanosomes/ml; groups L1 and L2 received 25µg/ml and 50µg/ml trypanosome lysate, respectively, in their media, while V1 and V2 received medium containing 25µg/ml and 50µg/ml VSGm, respectively. Groups T1 and T2 consisted of six flasks while each of the other groups had five flasks of confluent astrocyte monolayer. One flask in each group was not exposed to stimulant and served as a baseline control. Cells were harvested 2, 4, 6 and 8 hours after stimulation. In groups T1 and T2, there was an additional harvest at 24 hours after exposure.

**Table 4.1**

**The grouping of astrocyte cultures for stimulation**

Group	Stimulant	Dose
E	LPS	10 $\mu$ g/ml
T1	Trypanosomes	10 <sup>4</sup> /ml
T2	Trypanosomes	10 <sup>7</sup> /ml
L1	Trypanosome lysate	25 $\mu$ g/ml
L2	Trypanosome lysate	50 $\mu$ g/ml
V1	VSGm	25 $\mu$ g/ml
V2	VSGm	50 $\mu$ g/ml

The table shows the various stimuli and the dosages at which they were used to stimulate astrocytes *in vitro*.

VSGm: membrane bound form of variable surface glycoprotein.

LPS: Lipopolysaccharide

Trypanosomes, in groups T1 and T2, were alive and actively motile throughout the duration of the experiment.

#### **4.2.7. Harvesting cells for RNA extraction**

At each time point, the supernatant was removed and stored at  $-70^{\circ}\text{C}$  for subsequent analysis of secreted cytokines. The cells were washed twice with HBSS and then lysed with 2ml of denaturing solution (4mM guanidinium isothiocyanate, 25mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1M 2- $\beta$ -mercaptoethanol) and removed with a rubber policeman. The mixture of cells and denaturing solution was then transferred into glass-Teflon homogenisers and homogenised at  $4^{\circ}\text{C}$ , transferred into polypropylene tubes (Falcon) and stored at  $-70^{\circ}\text{C}$  until RNA was extracted.

Each flask of cells (used for any one time point for any stimulant) contained approximately  $5 \times 10^6$  astrocytes and so the amount of RNA from each flask was expected to be the same. To confirm this, polymerase chain reaction (PCR) was performed for actin transcripts (actin is a gene expressed by all cells) on all RNA extracts. In addition, amplification of the actin gene confirmed the integrity of the extracted RNA as well as verifying that it was amplifiable.

#### **4.2.8. Preparation of RNA**

RNA was prepared as described by Chomczynski and Sacchi (1987), using acid-guanidinium thiocyanate phenol chloroform extraction. The cell/denaturing solution homogenate was thawed. Sequentially, 0.1ml of 2M sodium citrate (pH 4.0), 1ml of water-saturated phenol and 0.2ml of chloroform/isoamyl alcohol (49:1) were added for each ml of denaturing solution in the homogenate, with thorough mixing after addition of each reagent. The final suspension was shaken vigorously for 10 seconds and cooled on ice for 15 minutes. Samples were centrifuged at 10,000g for 20 minutes at  $4^{\circ}\text{C}$ . The aqueous phase, containing RNA, was transferred to a fresh tube, mixed with

1ml of isopropanol, then placed at -20°C for at least 1 hour to precipitate RNA. Sedimentation at 10,000g for 20 minutes at 4°C was performed and the resulting RNA pellet was dissolved in 300µl of denaturing solution, transferred into a 1.5ml eppendorf tube and precipitated with two volumes of ethanol on dry ice for at least 30 minutes. After centrifugation for 10 minutes at 4°C, the RNA pellet was washed twice with 70% ethanol, sedimented by centrifugation, vacuum dried for 15 minutes, dissolved in 60µl of double distilled water and stored at -20°C. Five µl aliquots were heated at 72°C to denature RNA secondary structures and electrophoresed on a 1% agarose gel to identify the rRNA by separation of the 28S and 18S bands. This is indicative of the integrity of the rest of RNA molecules including the mRNA.

#### **4.2.9. Preparation of cDNA and amplification by polymerase chain reaction**

Primers for the different cytokines analysed were synthesised by Genosys (Cambridge, U.K.). The primers are designed to produce bands of different sizes depending on the cytokine being analysed (Brener, Tam, Nelson, Engleman, Suzuki, Fry and Larick, 1989); β-actin 540 base pairs (bp), TNFα 692bp, IFN-γ 460bp, macrophage inflammatory protein 1 (MIP-1) 267bp, IL-1α 625bp, IL-2 502bp, IL-4 399bp, IL-6 638bp and granulocyte-macrophage colony stimulating factor (GM-CSF) 368bp. Approximately 5µg of total RNA was lyophilised and resuspended in 9µl of annealing buffer (250mM KCl, 10mM Tris HCl, pH8.3, 1mM EDTA) with 0.5 µg of the 3'primer heated at 80°C for 3 minutes, incubated at 69°C for 20 minutes followed by a 20 minute cooling to 40°C. Fifteen ml of cDNA/dNTP buffer (24mM Tris HCl, pH 8.3, 16mM MgCl<sub>2</sub>, 18mM dithiothrietol, 0.4mM dNTPs) were added with 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco) and incubated at 43°C for 45 minutes. For analysis of the cDNA using PCR, 0.5µg of the 5'primer was added with 55µl of *Taq* polymerase buffer (100mM Tris HCl pH

8.0, 30mM  $(\text{NH}_4)_2\text{SO}_4$ , 3mM  $\text{MgCl}_2$ , 10mM mercaptoethanol), 20 $\mu\text{l}$  2.5mM dNTPs and 2 units of DNA polymerase (Promega). The reaction mixture was subjected to 35 cycles of 94°C (denaturation) for 1 minute, 60°C (annealing) for 2 minutes, 72°C (extension) for 3 minutes. To visualise the PCR products, 20 $\mu\text{l}$  of reaction mixture was electrophoresed through a 2% agarose gel and stained with ethidium bromide. Brain homogenates from CD1 mice have been reported to constitutively express mRNA for IL-1 $\alpha$  (Hunter *et al*, 1992c). For this reason, water was included as a negative control in all the PCRs performed, to rule out contamination, and unstimulated cells were taken to provide baseline levels. Spleen from *T.b brucei*-infected mice was used as positive control for all the cytokine transcripts analysed, except for GM-CSF for which a commercial positive control was purchased from Clontech Laboratories.

#### 4.2.10 Detection of cytokine protein in cultured astrocytes

Astrocytes were removed from a confluent flask by trypsinisation and seeded onto PLL coated coverslips contained in 24-well tissue culture plates, at a density of  $4 \times 10^4$  per coverslip. They were maintained in 50% astrocyte conditioned DMEM/10%FCS for the first 24 hours, after which the medium was changed to fresh DMEM/10%FCS and cells grown for a further 48 hours, to establish a stable confluent monolayer. The cells were then stimulated with either 10 $\mu\text{g}/\text{ml}$  LPS or 50 $\mu\text{g}/\text{ml}$  lysate or 50 $\mu\text{g}/\text{ml}$  VSGm, for 2, 4, 6 and 8 hours. At each time point, the cells were fixed with methanol at -20°C for 10 minutes and placed in PBS, pH 7.0 until the staining procedure could be carried out.

The coverslips were removed from the PBS containing wells, fixed onto glass slides with glassbond in ultra violet light (u.v. light activates glassbond) and incubated for 10 minutes with 5% bovine serum albumin (BSA) in Tris buffered saline (TBS), pH 7.0, containing 0.01% saponin to block non-specific binding and permeabilise cells for the antibodies. The cells were then

incubated with the primary antibodies, (rabbit anti-mouse TNF $\alpha$  and rabbit anti-mouse IL-1 $\alpha$  diluted 1 in 50 and 1 in 150 in TBS/1%BSA/0.01% saponin, respectively, Genzyme) for 1 hour at room temperature in a humid chamber. After washing with TBS twice (10 minutes each time), the secondary antibodies, goat anti-rabbit immunoglobulins (Vector Laboratories), diluted 1 in 200 for TNF and IL-1, were added and incubated for 30 minutes. They were then washed again in TBS and incubated with avidin-biotin alkaline phosphatase conjugate, diluted 1 in 50, for 30 minutes and washed again. For colour visualisation, the cells were incubated with levamisole, fast red and naphthol in veronal acetate buffer (i.e. naphthol/fast red) for 5 minutes. The cells were post-fixed in neutral buffered formalin, washed in water, counterstained with haematoxylin and coverslips mounted with aquamount.

#### **4.2.11. Detection of secreted cytokine protein in astrocyte supernatants**

An enzyme-linked immunosorbent assay, for mouse TNF, was used to test for secreted cytokines in the cell supernatants following exposure to various stimuli. Briefly, 96-well microtitre plates (Corning easy wash or Dynatech IV) were coated with 50 $\mu$ l/well of purified anti-mouse TNF antibodies at a concentration of 2 $\mu$ g/ml and incubated overnight at 4°C. The following morning, nonspecific binding was blocked by incubation with 50 $\mu$ l/well 10% FCS in PBS, pH 7.4, for 45 minutes at 37°C. The plates were rinsed three times in PBS containing 0.05% tween 20, followed by two washes of 3 minutes each.

Recombinant mouse tumor necrosis factor (TNF) purchased from Pharmigen was used as the standard cytokine. Ten serial double-dilution in PBS starting at 80ng/ml were prepared from the stock. The standard-cytokine dilutions and the neat supernatant samples were added (50 $\mu$ l/well) in duplicates, and incubated at 37°C for 2 hours. The plates were then washed in PBS/tween as before, incubated for 1 hour at room temperature with 50 $\mu$ l/well of

biotinylated mouse anti-TNF antibody (Pharmigen), at 4 $\mu$ g/ml, and washed again. Streptavidin peroxidase (Serotec), diluted 1 in 1,000 in 1% BSA/PBS/tween 20 was added (75 $\mu$ l/well) and incubated for 1 hour at 37°C and washed. The peroxidase substrate, 3,3', 5, 5' tetramethylbenzidine (TMB) was added at 100 $\mu$ l/well. Absorbencies were read at OD<sub>630</sub>, after 15 minutes.

### **4.3. RESULTS**

#### **4.3.1. Controls**

Messenger RNA transcripts for IL-1 $\beta$  were found in all astrocytes investigated, including those that were not exposed to any stimulant. This expression increased after exposure to the various stimuli as judged by the intensity of complementary DNA (cDNA) bands. Transcripts for the other cytokines were absent from non-stimulated control samples.

Using the PCR, all the positive controls were positive for mRNA transcripts for the cytokines studied. On electrophoresis, the actin PCR products from all the flasks produced bands of approximately same intensity. Therefore, PCR for cytokine transcripts was used semiquantitatively, i.e., a difference in band intensity was taken to be indicative of a difference in the amounts of transcripts expressed.

LPS at a concentration of 10 $\mu$ g/ml stimulated astrocytes to express mRNAs for IL-1 $\alpha$ , IL-6, TNF $\alpha$ , MIP-1 and GM-CSF (Table 4.2). The thickness of the bands was used as an indication of the amount of cytokine transcript and designated either, -,  $\pm$ , +, ++ or +++ depending on the thickness of the bands. To illustrate this, the cytokine transcripts expressed for IL-1 $\alpha$ , MIP-1, GM-CSF and IL-1 $\beta$  are shown in Figure 4.1. Messenger RNA transcripts for all the cytokines studied, were detectable by 2 hours after exposure to LPS. Thereafter, the expression of transcripts increased, with those for IL-6 and IL-1 $\beta$  reaching ceiling levels by the 4<sup>th</sup> and 6<sup>th</sup> hours, respectively,

**Table 4.2****Cytokine transcripts expressed by astrocytes following stimulation with lipopolysaccharide**

Cytokine	dose	Time in hours				
	$\mu\text{g/ml}$	C	2	4	6	8
IL-1 $\alpha$	10	-	+	++	+++	+
IL-1 $\beta$	10	$\pm$	$\pm$	+	++	++
IL-6	10	-	+	++	++	++
TNF $\alpha$	10	-	+	++	++	+
GM-CSF	10	-	+	++	++	+++
MIP-1	10	-	+	++	++++	+++

The table shows results of the semiquantitative PCR judging from the thickness of the bands produced after electrophoresis on 2% agarose gel and staining with ethidium bromide. The numbers represent time in hours after addition of LPS.

C: Unexposed cells which provided the baseline levels of cytokine transcript;

-: No band

$\pm$ : Faint band

+: Distinctly visible band

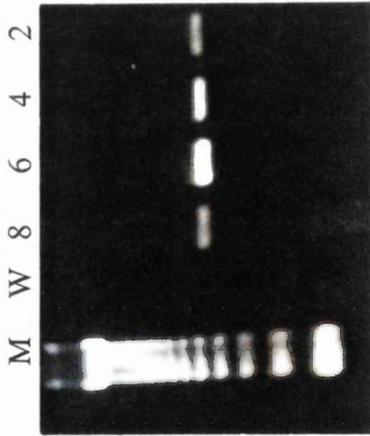
++: Fairly thick band

+++ : Thick band

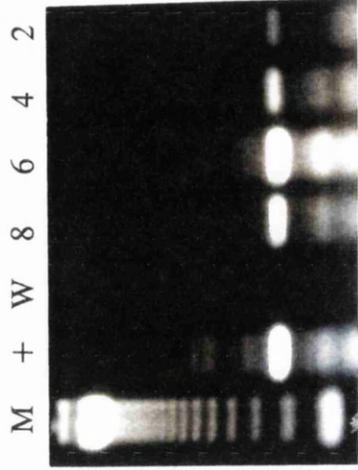
++++: Very thick band

### Figure 4.1

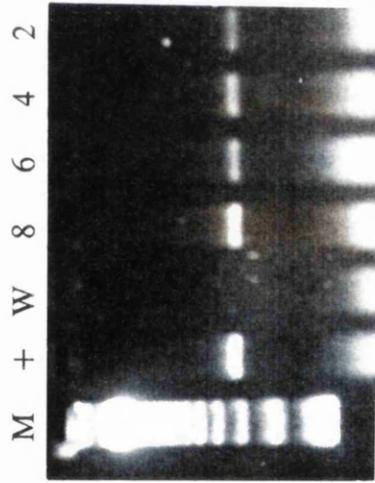
Gel electrophoresis showing cytokine transcripts expressed by astrocytes exposed to LPS (group E): a) IL-1 $\alpha$ , b) MIP-1, c) GM-CSF and d) IL-1 $\beta$ . 2, 4, 6 and 8 is time in hours after introduction of LPS. W = water which was used as negative control; + = positive control and M = molecular weight marker, 123bp ladder (Gibco).



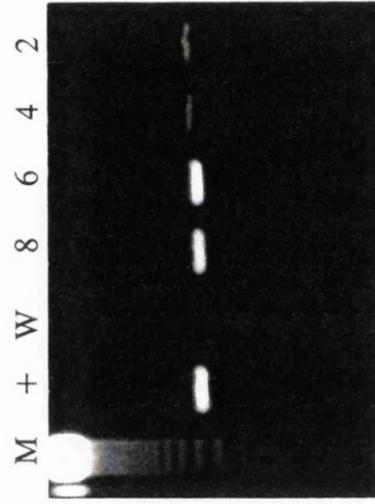
IL-1 $\alpha$   
(625bp)



MIP-1  
(267bp)



GM-CSF  
(238bp)



IL-1 $\beta$   
(625bp)

while those of GM-CSF increased steadily throughout the study period. However, mRNAs for IL-1 $\alpha$  and MIP-1 peaked at the 6<sup>th</sup> hour showing a decrease by the 8<sup>th</sup> hour. Transcripts for TNF $\alpha$  seem to have peaked between the 4<sup>th</sup> and the 6<sup>th</sup> hour and were showing a decline by the 8<sup>th</sup> hour (Table 4.2).

It is noteworthy that, on a number of occasions, more than one band was evident after amplification of specific cytokine transcripts. This may be as a result of dimerisation of the specific cDNA amplified, or an amplification of a non-specific cDNA sequence, since the conditions used for synthesis and amplification of all the cDNAs were identical, and not necessarily the optimal ones for some of the cytokines tested.

#### **4.3.2. Stimulation with live trypanosomes**

10<sup>4</sup> trypanosomes/ml induced transient expression of IL-1 $\alpha$  and IL-6, detectable 2 and 4 hours after exposure, disappearing thereafter. At the same time, transient low levels of TNF $\alpha$  were detected only on the sixth hour of exposure (Table 4.3 and Figures 4.2a). A slight increase in the amounts of IL-1 $\beta$  transcripts was noticed at 2 and 8 hours after exposure, falling to baseline levels in the 4<sup>th</sup> and 6<sup>th</sup> hours of study. Transcripts for MIP-1 were detectable from the 2<sup>nd</sup> hour after exposure showing a slight increase by the 4<sup>th</sup> hour, which was increasing even at the 24<sup>th</sup> hour (Figure 4.2b). This concentration of trypanosomes did not induce expression of GM-CSF.

Increasing the density of trypanosomes to 10<sup>7</sup>/ml, stimulated the expression of TNF $\alpha$ , IL-6 (Figure 4.2a and b) and GM-CSF (Figure 4.3b), transcripts from the 2<sup>nd</sup> hour of exposure. While the levels of GM-CSF transcripts remained the same, those of IL-6 and TNF $\alpha$  increased on the 6<sup>th</sup> and 8<sup>th</sup> hours, respectively, and remained at this level to the end of the 24-hour study period (Table 4.3). Similarly, MIP-1 transcripts were detectable after 2 hours of exposure to 10<sup>7</sup>/ml trypanosomes, exhibiting a peak at the 6 hour and another smaller peak at the 24 hour time point (Table 4.3).

**Table 4.3****Cytokine transcripts expressed by astrocytes after stimulation with live trypanosomes**

Cytokine	Dosage tryps/ml	Time in hours					
		C	2	4	6	8	24
IL-1 $\alpha$	10 <sup>4</sup>	-	+	+	-	-	-
	10 <sup>7</sup>	-	-	++	++	++	-
IL-1 $\beta$	10 <sup>4</sup>	±	+	±	±	+	-
	10 <sup>7</sup>	±	+	++	++	++	++
IL-6	10 <sup>4</sup>	-	+	+	-	-	-
	10 <sup>7</sup>	-	+	+	++	++	++
TNF $\alpha$	10 <sup>4</sup>	-	-	-	±	-	-
	10 <sup>7</sup>	-	+	+	+	++	++
GM-CSF	10 <sup>4</sup>	-	-	-	-	-	-
	10 <sup>7</sup>	-	+	+	+	+	+
MIP-1	10 <sup>4</sup>	-	+	++	++	++	++++
	10 <sup>7</sup>	-	+	++	++++	++	+++

The table shows results of the semiquantitative PCR judging from the thickness of the bands produced after electrophoresis on 2% agarose gel and staining with ethidium bromide. The numbers represent time in hours after addition of trypanosomes. C: Unexposed cells which provided the baseline levels of cytokine transcript;

-: No band

±: Faint band

+: Distinctly visible band

++: Fairly thick band

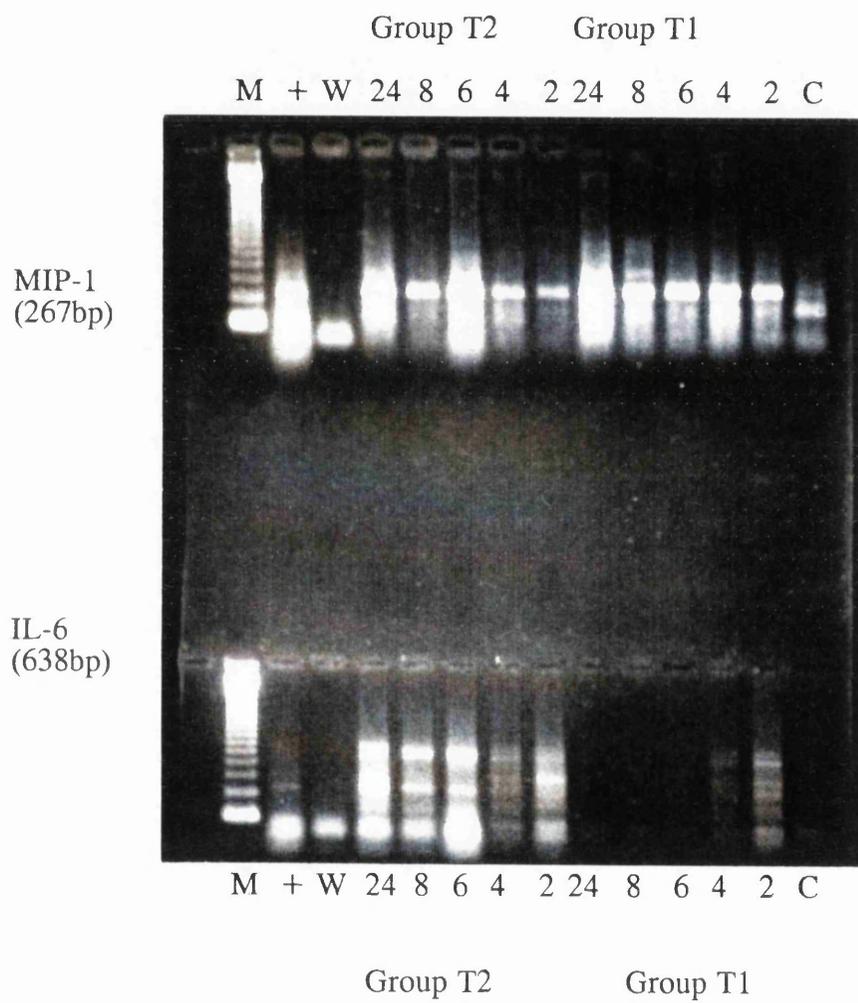
+++ : Thick band

++++: Very thick band

## Figure 4.2

Gel electrophoresis showing transcripts for a) IL-1 $\alpha$  and TNF $\alpha$ , b) MIP-1 and IL-6, extracted from astrocytes in group T1, stimulated with 10<sup>4</sup>/ml (right), and group T2 stimulated with 10<sup>7</sup>/ml (left) live trypanosomes. 2, 4, 6, 8 and 24 represent the time in hours after addition of trypanosomes. C = transcripts from cells that were not exposed to stimulant; W = water that was used as negative control; + = positive control; M = molecular weight marker, 123bp ladder (Gibco).

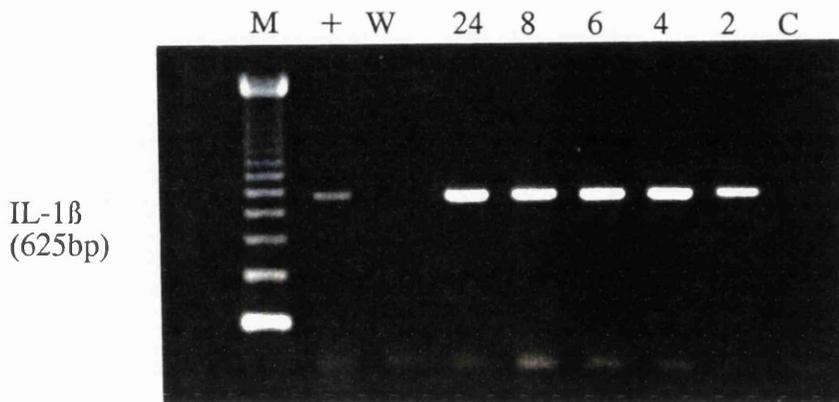
b)



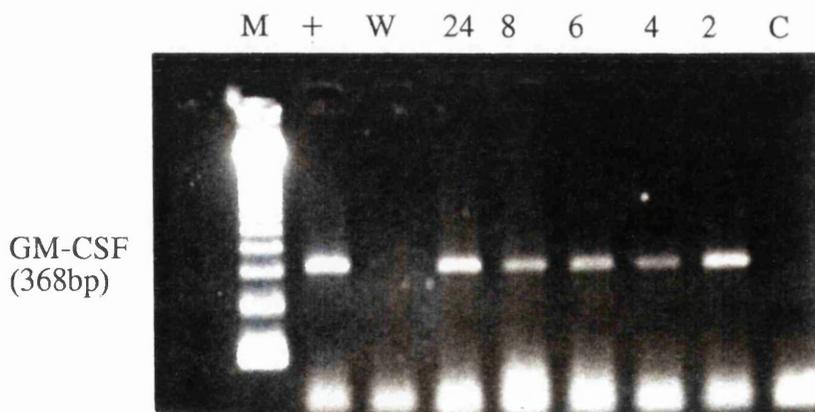
### Figure 4.3

Gel electrophoresis showing amplified transcripts for a) IL-1 $\beta$  and b) GM-CSF expressed by astrocytes in group T2, exposed to 10<sup>7</sup>/ml trypanosomes. The numbers represent the time in hours after addition of trypanosomes. C = transcripts from cells that were not exposed to stimulant; W = water that was used as negative control; + = positive control and M = molecular weight marker, 123bp ladder (Gibco).

a)



b)



The higher density of trypanosomes upregulated the expression of IL-1 $\beta$  from the 2<sup>nd</sup> hour, showed an increase on the 4<sup>th</sup> hour after exposure, and remained high for the rest of the study period (Table 4.3 and Figure 4.3a). IL-1 $\alpha$  transcripts induced by 10<sup>7</sup> trypanosomes/ml were detectable 4 hours after exposure, remained at the same level at the 6 and 8 hour sampling but had disappeared by 24 hours (Table 4.3 and Figure 4.2a).

#### **4.3.3. Stimulation with whole trypanosome lysate**

The whole trypanosome lysate also induced expression of mRNAs for IL-1 $\alpha$ , IL-6, TNF $\alpha$ , MIP-1 and GM-CSF, and upregulated the expression IL-1 $\beta$  transcripts (Table 4.4 and Figures 4.4 to 4.6). Transcripts for IL-6 and MIP-1 were detected 2 hours after exposure to 25 $\mu$ g/ml trypanosome lysate, while those of GM-CSF and IL-1 $\alpha$  were detectable at 4 and 6 hours, respectively (Table 4.4). Thereafter, there was an increase in the levels of IL-6 and MIP-1 at 4 and 8 hours, respectively, with those of IL-6 remaining at this level for the rest of the study period. In contrast, levels of GM-CSF remained the same while those of IL-1 $\alpha$  declined at the 8<sup>th</sup> hour of study (Table 4.4). Expression of IL-1 $\beta$  was upregulated, reaching a peak between 4 and 6 hours after exposure and showing a decline at the 8<sup>th</sup> hour of the study. Trypanosome lysate, at this concentration, did not induce expression of TNF $\alpha$  mRNA transcripts even after 8 hours of exposure (Table 4.4).

Increasing the concentration of trypanosome lysate to 50 $\mu$ g/ml, hastened the expression of GM-CSF and IL-1 $\alpha$ , so that transcripts were detectable at 2 and 4 hours, respectively, with those GM-CSF peaking at the 4<sup>th</sup> hour while those of IL-1 $\alpha$  displayed a drop at the 6<sup>th</sup> hour increasing again at the 8<sup>th</sup> hour. It also upregulated the expression of IL-1 $\beta$  from the 2<sup>nd</sup> hour of study, with levels remaining the same for the rest of the study period. Transcripts for IL-6 and MIP-1 were detectable at 2 hours and showed an increase at 4 hours. While the levels of MIP-1 peaked at the 6<sup>th</sup> hour, those of IL-6 reached a peak between

**Table 4.4**

**Cytokine transcripts expressed by astrocytes following stimulation with whole trypanosome lysate**

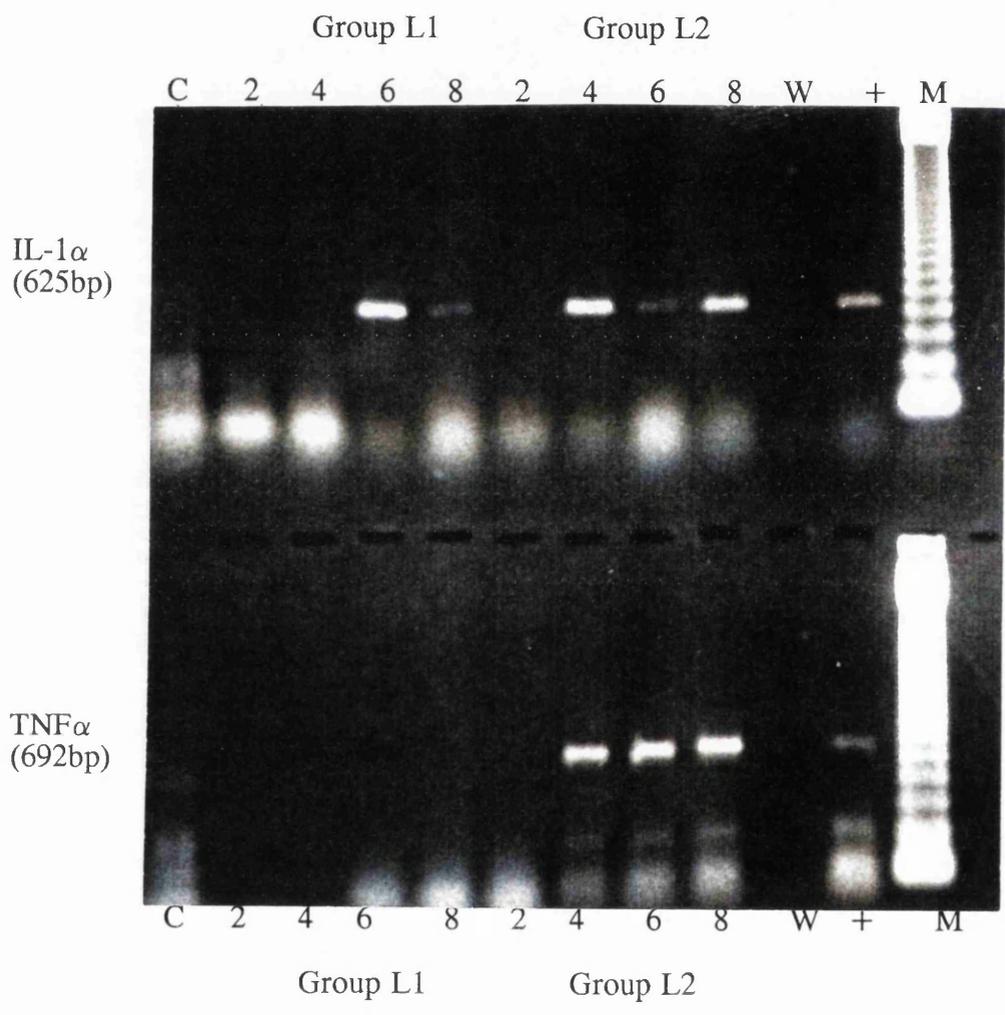
Cytokine	Dosage	Time in hours				
	$\mu\text{g/ml}$	C	2	4	6	8
IL-1 $\alpha$	25	-	-	-	++	+
	50	-	-	++	+	++
IL-1 $\beta$	25	$\pm$	+	++	++	+
	50	$\pm$	++	++	++	++
IL-6	25	-	+	++	++	++
	50	-	+	++	++	-
TNF $\alpha$	25	-	-	-	-	-
	50	-	-	++	++	++
GM-CSF	25	-	-	+	+	+
	50	-	+	++	+	+
MIP-1	25	-	+	+	+	++
	50	-	+	++	+++	++

The table shows results of the semiquantitative PCR judging from the thickness of the bands produced after electrophoresis on 2% agarose gel and staining with ethidium bromide. The numbers represent time in hours after addition of trypanosome lysate. C: Unexposed cells which provided the baseline levels of cytokine transcript;

- : No band
- $\pm$ : Faint band
- +: Distinctly visible band
- ++: Fairly thick band
- +++ : Thick band

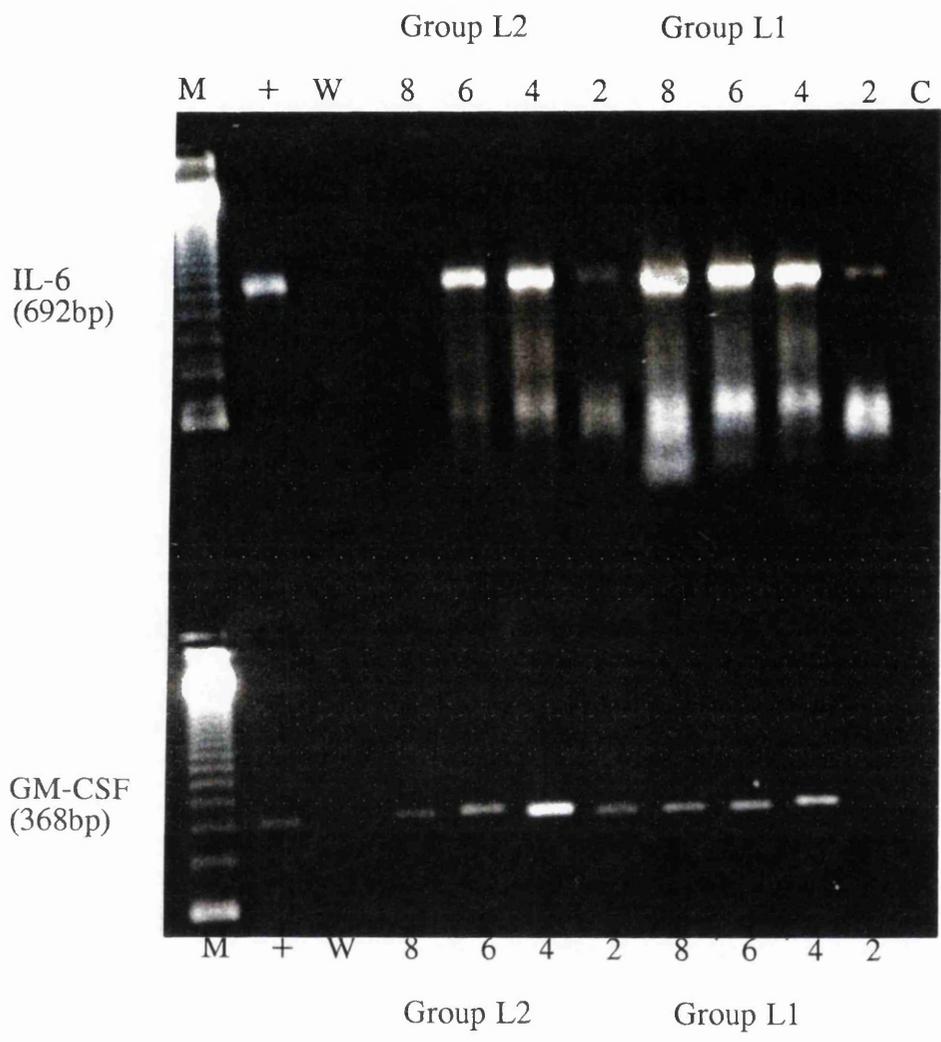
#### Figure 4.4

Transcripts for IL-1 $\alpha$  and TNF $\alpha$  expressed by astrocytes in group L1, which were exposed to trypanosome lysate at 25 $\mu$ g/ml (left) and from group L2, exposed to 50 $\mu$ g/ml (right). 2, 4, 6 and 8 represent the time in hours after addition of trypanosome lysate. C = transcripts from cells that were not exposed to stimulant; W = water that was used as negative control; + = positive control; M = molecular weight marker, 123bp ladder (Gibco).



### Figure 4.5

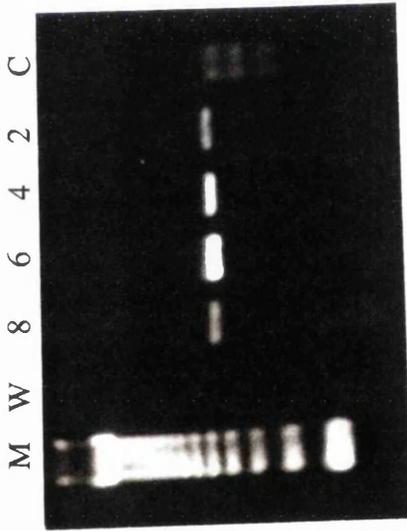
Transcripts for IL-6 and GM-CSF expressed by astrocytes in group L1, exposed to trypanosome lysate at 25 $\mu$ g/ml (right) and astrocytes from group L2, exposed to 50 $\mu$ g/ml (left). 2, 4, 6 and 8 represent the time in hours after addition of trypanosomes lysate. C = transcripts from cells that were not exposed to stimulant; W = water that was used as negative control; + = positive control; M = molecular weight marker, 123bp ladder (Gibco).



#### Figure 4.6

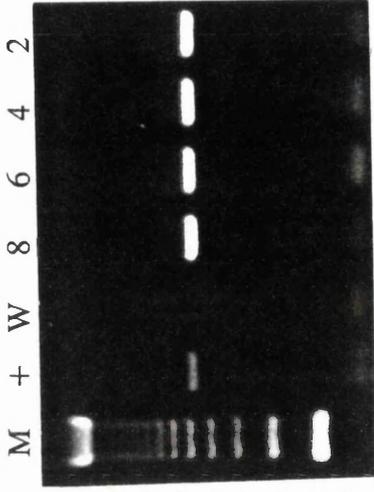
Cytokine transcripts expressed by astrocytes after exposure to 25 $\mu$ g/ml, group L1, and 50 $\mu$ g/ml, group L2, trypanosome lysate. Panels a and b show the transcripts for IL-1 $\beta$ , while panel c shows transcripts for MIP-1. 2, 4, 6 and 8 represent the time in hours after addition of trypanosome lysate. C = transcripts from cells that were not exposed to stimulant; W = water that was used as negative control; + = positive control and M = molecular weight marker, 123bp ladder (Gibco).

Group L1



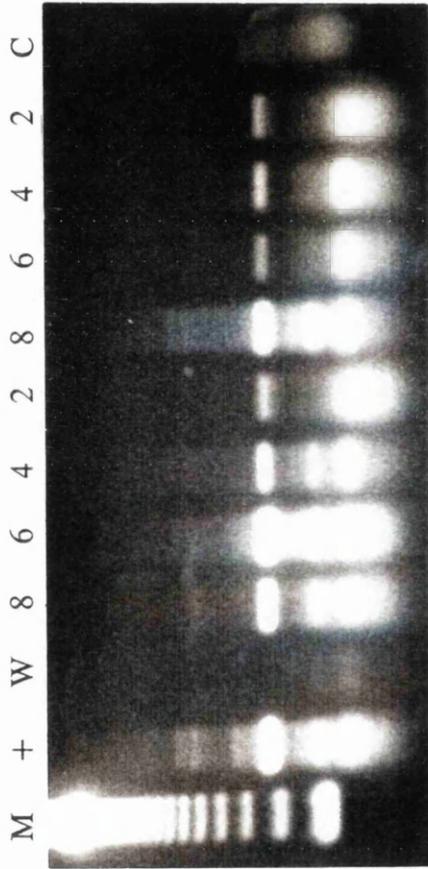
IL-1B  
(625bp)

Group L2



IL-1 $\beta$   
(625bp)

Group L2



MIP-1  
(267bp)

Group L1

4 and 6 hours after exposure and had disappeared by the 8<sup>th</sup> hour of study. Trypanosome lysate at 50 $\mu$ g/ml induced expression of TNF $\alpha$  transcripts, which were detected from the 4<sup>th</sup> hour and remained at the same level throughout the study period (Table 4.4).

Translation of cytokine message into protein, for both IL-1 $\alpha$  and TNF $\alpha$  following exposure to 50 $\mu$ g/ml trypanosome lysate, was detectable by immunocytochemistry from the 4<sup>th</sup> hour through to the 8<sup>th</sup> hour of study, with cells staining more intensely for IL-1 $\alpha$  than for TNF $\alpha$ . There was no protein detectable in the unstimulated cells or in the cells stimulated for 2 hours (Figure 4.7). The protein was diffusely distributed in the cytoplasm of most cells but was more intense in the perinuclear zone (Figure 4.8). In contrast to IL-1 $\alpha$  and TNF $\alpha$ , IL-6 was detectable in the first two hours after stimulation, but this declined and was only faintly detectable on the 6<sup>th</sup> and 8<sup>th</sup> hours of study (data not shown).

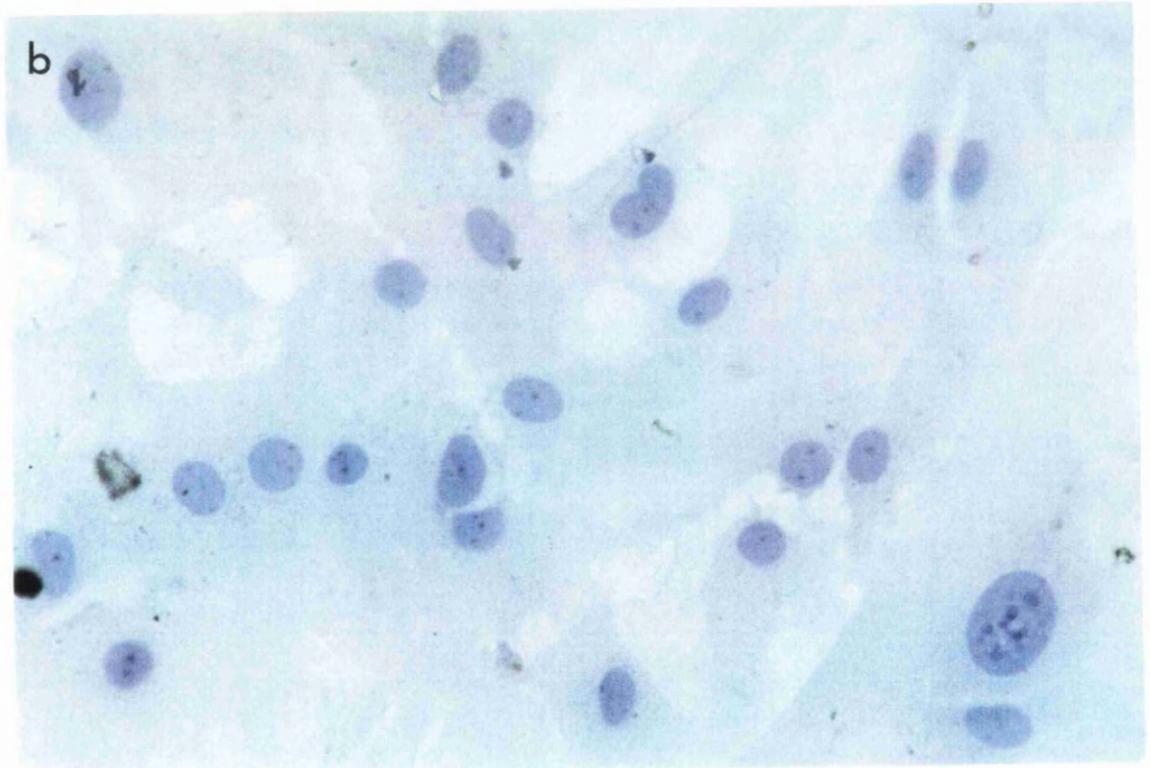
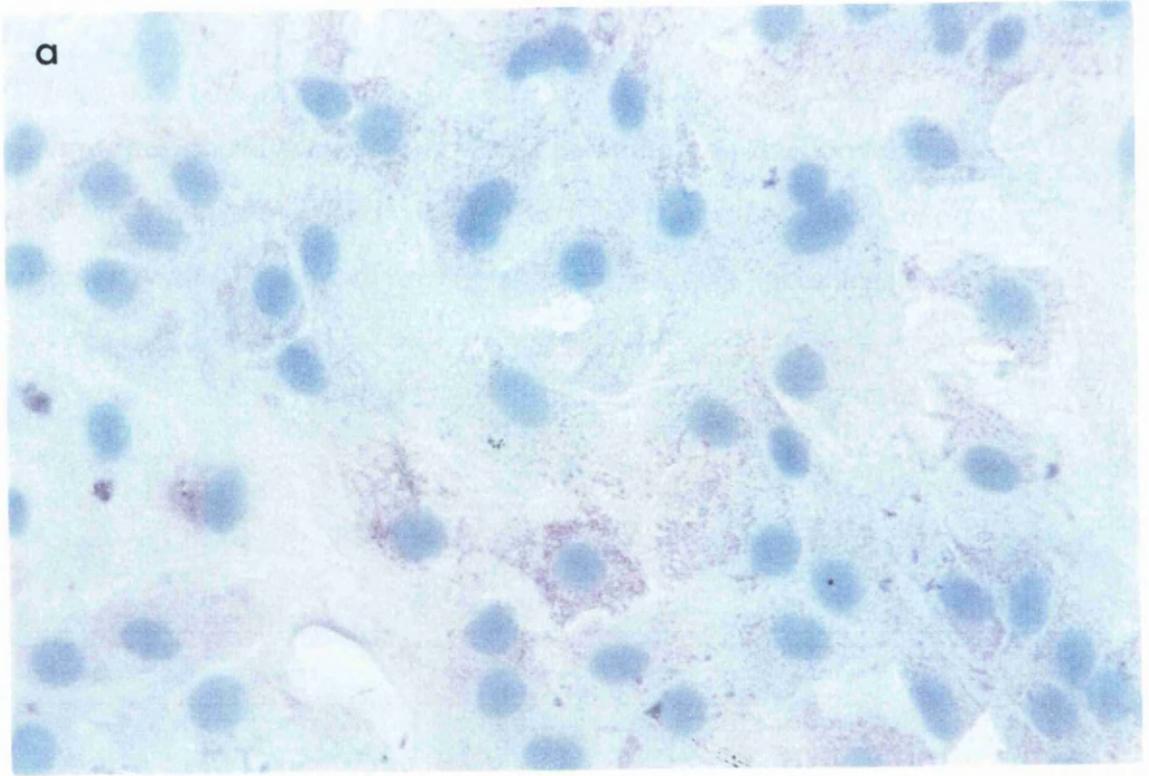
An attempt was made to determine whether TNF $\alpha$  protein was secreted and to quantify the amounts, by an enzyme-linked immunosorbent assay (ELISA) (Pharmingen, 1993). Although this technique detected as little as 0.625ng/ml of TNF protein in the standard, the levels of this cytokine in the astrocyte supernatants from all the stimuli, used in this study, were undetectable by this method.

#### **4.3.4. Stimulation with the membrane-bound form of variable surface glycoprotein**

Following exposure to 25 $\mu$ g/ml VSGm, induction of transcripts for IL-1 $\alpha$  and MIP-1, as well as an increase in the expression of IL-1 $\beta$  were detected by the 2<sup>nd</sup> hour, while expression of IL-6, TNF $\alpha$  and GM-CSF occurred by the 4<sup>th</sup> hour (Table 4.5). However, this induction seems to have been transient, with complete disappearance of IL-1 $\beta$  by the 4<sup>th</sup> hour of study, and of IL-1 $\alpha$ , GM-CSF and TNF $\alpha$  by the 8<sup>th</sup> hour of study. There was a

#### **Figure 4.7**

Micrographs showing the presence of cytokine protein in astrocytes from group L2, which were stimulated with 50 $\mu$ g/ml of trypanosome lysate. The pink colour shows the presence of IL-1 $\alpha$  after 8 hours of exposure (a). Control astrocytes that were not exposed to stimulation did not stain for IL-1 $\alpha$  (b). Naphthol red is used as chromogen and the nuclei are stained with haematoxylin. x200



### **Figure 4.8**

Astrocytes show the perinuclear position of the cytokine protein. The astrocytes were immunostained for IL-1 $\alpha$  after 8 hours of exposure to 50 $\mu$ g/ml trypanosome lysate. Naphthol red was used as the chromogen, and haematoxylin as the counterstain. x400

**Table 4.5**

**Cytokine transcripts expressed by astrocytes after stimulation with the membrane-bound form of variable surface glycoprotein**

Cytokine	Dose	Time in hours				
	$\mu\text{g/ml}$	C	2	4	6	8
IL-1 $\alpha$	25	-	+	+	+	-
	50	-	$\pm$	+	+	-
IL-1 $\beta$	25	$\pm$	+	-	-	-
	50	$\pm$	-	-	-	-
IL-6	25	-	-	$\pm$	+	$\pm$
	50	-	+	$\pm$	-	-
TNF $\alpha$	25	-	-	+	+	-
	50	-	+	-	-	-
GM-CSF	25	-	-	+	++	-
	50	-	-	-	$\pm$	-
MIP-1	25	-	$\pm$	+	++	+
	50	-	$\pm$	$\pm$	++	+

The table shows results of the semiquantitative PCR judging from the thickness of the bands produced after electrophoresis on 2% agarose gel and staining with ethidium bromide. The numbers represent time in hours after addition of membrane bound form of variable surface glycoprotein. C: Unexposed cells which provided the baseline levels of cytokine transcript;

- : No band
- $\pm$ : Faint band
- +: Distinctly visible band
- ++: Fairly thick band

decrease in the expression of IL-6 and MIP-1 mRNA by the 8<sup>th</sup> hour of study.

Increasing the concentration of VSG inhibited the expression of IL-1 $\beta$  and decreased the duration of transcript expression for IL-6 and TNF $\alpha$  by 1 hour and of GM-CSF by 2 hours, although IL-6 and TNF $\alpha$  transcripts were detected at an earlier stage. However, it did not affect the duration of IL-1 $\alpha$  and MIP-1 expression (Table 4.5).

#### **4.3.5. Detection of secreted cytokines in the astrocyte supernatants**

Astrocyte supernatants, from each of the stimulant used in this study, were tested for the presence of secreted TNF $\alpha$  protein by ELISA (Chapter 2). Although all the standard cytokine dilutions, the lowest of which was 1.25ng/ml, gave positive results, no TNF $\alpha$  was detected from any of the astrocyte supernatants.

#### **4.4. DISCUSSION**

For the first time, trypanosomes and trypanosome-derived products have been shown to induce cultured astrocytes to produce the cytokines IL-1 $\alpha$  and - $\beta$ , IL-6, TNF $\alpha$ , GM-CSF, and MIP-1. Amongst the cytokines studied here, the production of IL-1 $\alpha$  and TNF $\alpha$  mRNA transcripts and protein are possibly the most significant, considering their key role in the initiation of meningeal inflammation (Ramilo *et al*, 1990; Saukkonen *et al*, 1990).

The results showed that all cultured astrocytes expressed the mRNA transcripts for IL-1 $\beta$ , including, the cells that were not exposed to any stimulant. While this may result from something in the culture medium selectively stimulating expression of this cytokine transcript, it is likely that astrocytes from NIH mice constitutively express the mRNA for IL-1 $\beta$ . Other researchers have reported constitutive expression of colony-stimulating factor 1 (CSF-1) in cultured astrocytes (Lee *et al*, 1993b) and IL-1 $\alpha$  transcripts in the brains of CD 1 mice (Hunter *et al*, 1992c).

Trypanosomes, whole trypanosome lysate and VSG were found to induce astrocytes, in culture, to express mRNAs for the cytokines IL-1 $\alpha$  and - $\beta$ , TNF $\alpha$ , IL-6, GM-CSF and MIP-1. Live trypanosomes and whole trypanosome lysate were potent stimuli, inducing, expression of these cytokines for longer durations than the less potent VSGm. This suggested that other parts of trypanosomes, or factors produced by these parasites, might be additive to VSGm in upregulating the expression of cytokine transcripts.

The results of immunocytochemistry showed that translation of the cytokine message into protein was rapid with the detection of translated protein, in the three cytokines investigated this way, namely IL-1 $\alpha$ , IL-6 and TNF $\alpha$ , coinciding with the time the message was detected. It is possible that there was a time lapse (of less than two hours) between the mRNA gene expression and translation of the message into protein but, due to the timing of these experiments, the lapse was not detectable. The decrease in the IL-6 protein in the 6<sup>th</sup> and 8<sup>th</sup> hours might mean that this cytokine was rapidly secreted from the cells into the surrounding environment. Unfortunately, the attempt to demonstrate the presence of secreted cytokine in the cell supernatants, by ELISA, gave negative results. There are a number of possible reasons why detection of secreted cytokine by ELISA might fail. 1) The cytokine protein in supernatants from the cell cultures might have been very dilute since 10ml of the culture medium was used to feed only  $5 \times 10^6$  astrocytes. Other researchers using VSG- and LPS- and concanavalin A (Con A)-stimulated macrophages (Mathias *et al*, 1990; Dr. E.Devaney, personal communication) used 1ml of medium to feed  $10^6$  cells, and successfully detected secreted cytokines using this technique. 2) The supernatants in this study were stored for long durations and the pH of the medium had changed to above 8 by the time the ELISA was performed; these circumstances might have denatured the cytokines. 3) It is possible that under the *in vitro* conditions, the cytokine studied, TNF, was not secreted

during the period of study.

The cytokines produced by cultured astrocytes in response to whole trypanosomes, their lysate, or the VSGm *in vitro* have been shown to induce important biological changes in various cells, including, blood vascular endothelial cells (Montovani and Dejana, 1989), lymphocytes (Kasahara *et al*, 1991; Merrill *et al*, 1992), macrophages (Broudy, Kaushansky, Harlan and Adamson, 1987; Sieff, Niemeyer, Mentzer and Faller, 1988; Kasahara *et al*, 1991), microglia (Hurwitz *et al*, 1992; Lee, *et al*, 1993a; 1993b) and astrocytes themselves (Fontana *et al*, 1981; Lieberman *et al*, 1989; Yong *et al*, 1991; Merrill *et al*, 1992). For example, TNF $\alpha$  and IL-1 $\alpha$  not only induce proliferation of astrocytes but also expression of MHC class II antigens and intercellular adhesion molecules (McCarron *et al*, 1986). These cytokines exert similar effects on macrophages (Rouzer and Cerami, 1980), endothelial cells (Montovani and Dejana, 1989), microglia (Ramilo *et al*, 1990; Saukkonen *et al*, 1990; Hurwitz *et al*, 1992; Lee *et al*, 1993a; 1993b), and lymphocytes (Selmaj *et al*, 1990). IL-1 $\alpha$  and TNF- $\alpha$  have also been found to induce production of cytokines such as IL-1 $\alpha$ , TNF $\alpha$ , IL-2 and IL-6 in astrocytes, microglia (Ramilo *et al*, 1990; Saukkonen *et al*, 1990; Chen, Hinton, Apuzzo and Hofman, 1993), endothelial cells (Montovani and Dejana, 1990), macrophages and lymphocytes (Broudy *et al*, 1987; Sieff *et al*, 1988; Selmaj *et al*, 1990; Kasahara *et al*, 1991; Merrill *et al*, 1992).

The demonstration, *in vitro*, that trypanosomes can induce astrocytes to produce cytokines complements the *in vivo* studies in Chapter 3, where astrocyte activation (as judged by the intensity of GFAP staining) occurred early in the course of infection, prior to the infiltration of inflammatory cells into the CNS of *T.b.brucei*-infected mice. Indeed, induction of mRNA transcripts for some of these cytokines, including, TNF, IL-1, and MIP-1, in addition to IL-4 and IFN $\gamma$  in the brains of *T. brucei*-infected mice have been reported to coincide with astrocyte activation (Hunter *et al*, 1991). The results of the current *in vitro*

study suggest that astrocytes may produce the cytokines that start the cascade of events, leading to the development of the CNS inflammation observed in late-stage trypanosomiasis (Chapter 1).

It would appear likely that the six cytokines found to be produced by trypanosome-stimulated astrocytes, in this study, might have an important role in the initiation and propagation of the CNS pathology found in *T.b.brucei* infected mice (Jennings *et al*, 1989) and in sleeping sickness patients (Adams *et al*, 1986; Haller *et al*, 1986). For instance, IL-1 upregulates expression of intercellular adhesion molecule 1 (ICAM-1) and endothelial leukocyte adhesion molecule 1 (ELAM-1) which alter the adhesive properties of the vascular endothelium, and initiate leukocyte extravasation. IL-1 also induces the endothelial cells to produce chemotactic cytokines such as IL-8, macrophage chemotactic protein (MCP) and colony-stimulating factors (CSFs) which lead to leukocyte recruitment and proliferation (Broudy *et al*, 1987; Sieff *et al*, 1988).

IL-1 is a polyclonal activator of lymphocytes inducing production of other cytokines, amongst which, the production of IL-2, and the expression of IL-2 receptors, are of paramount importance (Durum, Schmidt and Oppenheim, 1985). The action of IL-2 on the induced IL-2 receptors stimulates proliferation of lymphocytes, differentiation into antibody-producing plasma cells, modulates expression of IL-2 receptor and downregulates the production of IL-1, thereby providing a form of negative feedback mechanisms for IL-2 activity and IL-1 production, respectively (Walldmann, 1993). Thus, IL-1 might contribute to the increase in the number of lymphocytes and the occurrence of plasma cells in the CNS of late-stage sleeping sickness patients (Adams *et al*, 1986; Haller *et al*, 1986) and of *T.b.brucei*-infected mice (Murray *et al*, 1974; Jennings *et al*, 1989). Other important activities of IL-1, include, augmentation of natural killer (NK)-mediated cytotoxicity, induction of prostaglandin release, chemotaxis and tumour cell lysis by macrophages; and induction of fever (Durum *et al*, 1985);

these are also properties of activated macrophages. IL-1 production in the brain may thus account for the presence of activated macrophages in the CNS of late-stage trypanosomiasis (Poltera, 1980; Schultzberg *et al*, 1988).

TNF $\alpha$  induces migration of endothelial cells (ECs) but inhibits their proliferation, stimulates production of chemotactic cytokines and CSFs (Seelentag *et al*, 1987), augments the expression of MHC class I expression (Pober *et al*, 1987b) and has also been reported to induce migration of monocytes across the blood-brain barrier (Saukkonen *et al*, 1990). Treatment of cultures of human fetal astrocytes with TNF $\alpha$  induces expression of various adhesion molecules, including, E-selectin and IG9 message and protein (both of which are monocyte adhesion molecules), vascular cell adhesion molecule 1 (VCAM-1) (Hurwitz *et al*, 1992) and ICAM-1 (Sato, Kastrukoff and Kim, 1991; Frohman *et al*, 1989). This implies that once monocytes and lymphocytes have crossed the blood-brain barrier, their migration to sites of inflammation within the CNS parenchyma might be facilitated by the expression of adhesion molecules on astrocytes (Hurwitz *et al*, 1992). It is also possible that astrocytes respond to the TNF they (astrocytes) and other inflammatory cells produce during trypanosome infections by expressing adhesion molecules which in turn enhance the spread of inflammatory cells in the CNS.

GM-CSF (and G-CSF) induce migration and proliferation of endothelial cells but do not stimulate their proinflammatory/prothrombotic properties or functions associated with immune accessory activity. However, both these cytokines induce a pattern of responses in ECs that is similar to that induced by fibroblast growth factors (FGFs). Thus, they are potentially mitogenic and induce EC chemotaxis and proteinase synthesis which is required for cell migration and new vessel formation (Montovani and Dejana, 1989). The migration of ECs, in response to cytokines produced by astrocytes, might create leaks in the endothelial-brain barrier, allowing passage of cells and macromolecules, possibly even trypanosomes, into the Virchow Robin spaces to

form perivascular cuffs and produce conditions suitable for cellular proliferation and trypanosome replication. In addition, GM-CSF can cause proliferation of granulocyte-macrophage progenitor cells in bone marrow cultures, as well as the initial proliferation of multipotential stem cells in mouse fetal liver and adult marrow capable of forming mixed or pure erythroid, eosinophil or megakaryocyte colonies (Metcalf and Nicola, 1983). However, GM-CSF alone is inadequate in stimulating proliferation of terminal cell divisions in these pathways (Metcalf and Nicola, 1983), but can stimulate various functional activities of the mature post-mitotic granulocytic and macrophage cells such as increased RNA and protein synthesis (Burgess and Metcalf, 1980). Macrophages are stimulated to exhibit increased phagocytic and cytotoxic activity (Handman and Burgess, 1979), to increase production of prostaglandin E (PGE) (Kurland, Broxmeyer, Pelus, Bockman and Moore, 1978) and plasminogen activator (Lin and Gordon, 1979). GM-CSF also induces tumoricidal activity in human monocytes and macrophages (Grabstein, Urdal, Tushiski, Mochizuki, Price, Cantrell, Gillis and Conlon, 1986).

G- and GM-CSF seem to regulate both the proliferation of microglial cells (Giulian and Ingeman, 1988; Raivich, Gehrman and Kreutzberg, 1991) and their differentiation into macrophages (Suzumura, Sawada, Yamamoto and Marunouchi, 1990). GM-CSF induces the differentiation of intrinsic brain precursor cells into a population of microglia cells, which is intrinsically activated for antigen-presentation. Such microglia induce antigen-directed proliferation of  $T_H1$  and  $T_H2$  lymphocyte series (Fischer, Nitzgen, Germann, Degitz, Daubener and Hadding, 1993). Production of GM-CSF in the brain in late-stage trypanosomiasis might account for the proliferation and differentiation of microglia and antigen-directed proliferation of the infiltrating lymphocytes.

Interleukin 6 is a cytokine with pleiotropic biological functions, including, release of acute phase proteins, induction of differentiation of

activated B cells into immunoglobulin-secreting cells, stimulation of T cell activation, and cooperation with colony-stimulating factors in the induction of colony formation by haemopoietic progenitors (Van Snick, 1990). Human fetal astrocytes in culture are induced to produce IL-6 by IL-1 $\beta$  and to a lesser extent by TNF $\alpha$  (Yasukawa, Hirano, Watanabe, Muratani, Matsuda, Nakai and Kishimoto, 1987). The ability of astrocytes to produce IL-6 in response to cytokines (Frei *et al*, 1989) may represent an important mechanism by which resident brain cells, in particular astrocytes and microglia, enhance the local antibody response in infectious and autoimmune CNS disease, such as viral meningitis or encephalitis and multiple sclerosis, in which intrathecal synthesis of immunoglobulins is known to occur (Esiri, 1980; Resnick, Di Marzo-Veronese, Schupbach, Tourtellotte, Ho, Muller, Shapshak, Vogt, Groopman, Markham and Gallo, 1985; Vandvik, Skolenberg, Frosgren, Sternstedt, Jeansson and Norrby, 1985; Houssiau, Bukasa, Sindic, Van Damme and Van Snick, 1988; Frei, Leist, Meager, Gallo, Leppert, Zinkernagel and Fontana, 1988; Maimone, Gregory, Arnason and Reder, 1991). The finding, in this study, that astrocytes produce IL-6 in response to trypanosomes and their fractions (lysate and VSGm), might account for the presence of plasma cells and Russell-body containing plasma cells (Mott cells) in the CNS of late-stage sleeping sickness patients.

IL-6 causes proliferation and activation of T-lymphocytes. Therefore, this cytokine might be involved in enhancing the intracerebral T-lymphocyte responses against autoantigens and possibly trypanosomes, since lymphocyte proliferation and activation are characteristic findings in the CNS, during late-stage trypanosomiasis. The occurrence of autoantigens is evidenced by the presence of autoantibodies in the serum of *T.b. brucei*-infected mice (Hunter *et al*, 1992d). IL-6 also acts as a colony-stimulating factor (Montovani and Dejana, 1989) and might contribute to the migration of endothelial cells, and the proliferation of microglial and brain macrophages in the CNS during late stages

of trypanosomiasis.

Macrophage inflammatory protein 1 (MIP-1) is capable of inducing migration of monocytes across the blood-brain barrier (Saukkonen *et al*, 1989) and has been shown to be related to macrophage chemotactic protein 1 (MCP-1) which is monocyte specific (Yoshimura, Matsushima, Tanaka, Robinson, Appela, Oppenheim and Leonard, 1987). Production of MIP-1, by astrocytes, in the vicinity of endothelial cells in the brain increases expression of ICAM-1 and ELAM-1 and also the production of chemotactic cytokines (Montovani and Dejana, 1989), thus causing leukocyte extravasation and creating perivascular cuffs, which is a characteristic lesion observed in late-stage trypanosomiasis (Adams *et al*, 1986; Haller *et al*, 1982; Pepin and Milord, 1991).

IL-1 $\alpha$  and TNF $\alpha$  are additive in inducing EC responses, including migration, expression of adhesion molecules and production of cytokines, as well as procoagulant activity and neutrophil adhesion (Pober *et al*, 1987a; 1987b; Bevilacqua *et al* 1985; Bevilacqua *et al*, 1986a). In addition, IL-1 $\beta$  and TNF $\alpha$  stimulate astrocytes to produce colony-stimulating factor 1 (CSF-1), a cytokine that is responsible for development and proliferation of mononuclear phagocytes. CSF-1 induces hypertrophy and hyperplasia of microglia (Lee *et al*, 1993a; 1993b). However, CSF-1 is capable of down-regulating immune responses by inducing production of IL-1 inhibitors and down-regulating MHC class II antigens (Giulian, Woodward, Young, Krebs and Lachman, 1988).

Endothelial cells themselves are important producers of various mediators that regulate the haematopoietic system, the differentiation and proliferation of T and B cells, and the recruitment of leukocytes at sites of inflammation. Stimulation of endothelial cells with agents such as IL-1, TNF and LPS induces production of IL-1, TNF, CSFs, IL-6, PDGF and chemotactic factors (Libby, Ordovas, Birinyi, Auger, and Dinarello, 1986a; Libby, Ordovas, Auger, Robbins, Birinyi and Dinarello, 1986b; Miossec and Ziff, 1986;

Locksley, Heinzl, Shephard, Agosti, Eessalu, Aggarwal and Harlan, 1987; Broudy *et al*, 1987; Sieff *et al*, 1988; Libby, Warner and Friedman, 1988; Sironi, Brevario, Proserpio, Biondi, Vecchi, Van Damme, Dejana and Montovani, 1989). This suggests autocrine and/or paracrine circuits involving ECs and CSFs in the maintenance of the bone marrow microenvironment (Stern, Bank, Bawroth, Cassimeris, Kisiel, Fenton, Dinarello, Chess and Jaffe, 1985; Libby *et al*, 1986; Miossec and Ziff, 1986; Miossec, Cavendeer and Ziff, 1986; Locksley *et al*, 1987; Malone, Pierce, Falko and Metcalf, 1988). IL-1 and TNF produced by astrocytes during trypanosomal infections might stimulate ECs to produce CSFs, thus causing proliferation and differentiation of inflammatory cells, and enlarging the perivascular cuffs.

Many theories regarding the route of entry of trypanosomes into the CNS have been proposed and include free flow into the choroid plexus and paraventricular apparatus where the blood-brain barrier is incomplete, followed by passive distribution in the cerebrospinal fluid to all the Virchow Robin spaces, and migration of the trypanosomes from the perivascular areas to the rest of the CNS (Poltera, 1980; Schultzberg *et al*, 1988; Jennings *et al*, 1989). It might be possible for infectious agents such as trypanosomes, to activate both the astrocytes and endothelial cells at the blood-brain barrier and to utilise the resultant effects to gain entry into the CNS. Indeed, it has been reported that cytokines produced during bacterial meningitis breach the blood-brain barrier leading to invasion of the CNS by bacteria (Tuomanen, 1993). The cytokines shown to be produced by astrocytes in this study are capable of breaching the blood-brain barrier and may enable trypanosomes to invade the Virchow Robin spaces and the CNS parenchyma.

Production of CSFs by the astrocytes, microglia, endothelial cells and probably by the lymphocytes and macrophages in the formed perivascular cuffs might have autocrine and paracrine effects on all these cells causing further proliferation and local production of more cytokines, which would lead to

diffuse astrocyte activation and encephalitis, major features of the CNS pathology of African trypanosomiasis. IL-1 $\alpha$  is a polyclonal activator of lymphocytes inducing proliferation, production of cytokines and antibodies. Activated lymphocytes produce IFN- $\gamma$  which causes activation of astrocytes and expression of MHC class II antigens on their surfaces (McCarron *et al*, 1986; Yong *et al*, 1991). TNF $\alpha$ , IL-1 $\alpha$  and MIP-1 have been shown to invoke a unique set of pathological conditions, underscoring the fact that induction of brain damage in meningitis is the summation of multiple distinct inflammatory mechanisms (Saukkonen *et al*, 1990). Therefore, by producing the cytokines IL-1, TNF $\alpha$ , GM-CSF, IL-6 (which is colony stimulating), and MIP-1, astrocytes can cause migration of endothelial cells, expression of adhesion molecules, chemotaxis and proliferation of inflammatory cells at perivascular sites and within the CNS, and, at the same time, initiate antigen presentation within the CNS by inducing expression of MHC class II molecules. Therefore, there is a strong case that astrocytes might initiate and perpetuate the CNS pathological lesions observed in late-stage trypanosomiasis, possibly in association with endothelial, microglial and inflammatory cells.

It is surprising that neither neuronal degeneration nor necrosis or neutrophil infiltration into the CNS are prominent features of late-stage sleeping sickness since most of the cytokines shown to be produced by astrocytes, in this study, enhance natural killer activity, cytotoxicity, proliferation and activation of phagocytic cells, and neutrophil chemotaxis. It is possible that other cytokines produced by the astrocytes themselves or by other cells down-regulate the inflammatory reaction and prevent such degeneration in sleeping sickness. For instance, IL-1 induces production of IL-2 and expression of IL-2 receptors on lymphocytes, which in turn enhance cytotoxicity and natural killer activity. Interaction of IL-2 on the induced receptors down-regulates IL-2 receptor expression in a negative feedback manner (Walldmann, 1993). In addition, IL-1

stimulates prostaglandin production by macrophages which down-regulates the production of IL-1 (Beutler and Cerami, 1989). Indeed, production of prostaglandins, in the brain, during late-stage trypanosomiasis has been reported (Pentreath *et al*, 1990). Furthermore, cultured astrocytes produce IL-10 in response to trypanosomes (personal observations), a cytokine that has been reported to counteract the effects of IFN  $\gamma$ , suggesting a mechanism for downregulating inflammation. At the same time, GM-CSF downregulates the IFN $\gamma$ -induced MHC class II antigen expression on astrocytes (Lee *et al*, 1993b), thus interfering with the antigen presentation.

It has been observed that macrophages require an optimal level of *Plasmodium falciparum*-products for maximal TNF production, and that increasing the amounts of parasite products above this optimal level leads to lower TNF production (Picot, Peyron, Vuilez, Barbe, Marsh and Thomas 1990). It is possible that a similar mechanism is operational, in the CNS, during trypanosome infections, involving, TNF and other cytokines as well. Indeed, the results presented here show that increasing the concentration of VSGm for stimulating astrocytes *in vitro*, decreased gene expression for TNF $\alpha$ , IL-1 $\beta$ , IL-6 and GM-CSF, while the levels of IL-1 $\alpha$  and MIP-1 remained the same. Together, these events might lead to the lack of neuronal degeneration and the mild nature of the inflammatory reaction in the untreated, chronic *T.b. brucei* infections in mice (Chapter 3). A delicate balance of inflammatory and modulatory cytokines seems to exist in the CNS of *T.b. brucei*-infected mice, restricting the inflammatory reaction from developing to destructive proportions, thus sparing the neurones and brain tissue. It appears as if an active immune system is required to maintain this balance. If that is the case, then the generalised immunosuppression, which occurs in advanced late-stage disease, tips this balance producing the massive reaction and the degeneration of neuropil observed in terminal stages of the disease.

It must be emphasised that the data presented here probably identify

only a few factors in the scenario leading to the CNS inflammation in trypanosomiasis. Caution is, therefore, necessary in interpreting the cytokine data. This is due in part to their well-known redundancy and pleiotropism (Paul, 1989; Abbas, Litchman and Pober, 1991) but also to the unavailability of reagents needed to study the many other potential effector molecules such as neutral proteases, prostaglandins and other cytokines. In addition, the results discussed here are from an *in vitro* study and direct extrapolation to what might happen *in vivo* might be misleading, considering the presence of other cells *in vivo* that might produce mediators which could inhibit the production of and/or counteract the effects of the secreted inflammatory cytokines.

In conclusion, this study has shown that cultured astrocytes, exposed to trypanosomes and trypanosome fractions, produce the key cytokines that might be involved in the initiation and propagation of the meningeal inflammatory reactions leading to encephalitis. It is proposed that astrocytes respond to trypanosome invasion of the CNS by producing cytokines which recruit inflammatory cells into the perivascular spaces. Subsequently, the recruited inflammatory cells respond to the astrocyte-derived cytokines by becoming activated and producing cytokines themselves. The resultant cytokines then further stimulate both the intrinsic brain cells and the infiltrating inflammatory cells progressively aggravating leukocyte extravasation and later causing neuronal degeneration, leading to the severe meningoencephalitis that is found in advanced human African trypanosomiasis.

## **CHAPTER 5**

### **THE EFFECT OF EFLORNITHINE ON ASTROCYTE ACTIVATION *IN VIVO* AND *IN VITRO***

## 5.1 INTRODUCTION

Management of the late stages of sleeping sickness is hampered by the lack of safe, inexpensive therapeutic agents, resistance to and toxicity of the few available trypanocidal drugs, and a fatal post-treatment reaction in patients treated with melarsoprol, the only curative drug for the late stages of both the *gambiense* and the *rhodesiense* forms of the disease (de Raadt, 1984; Gutteridge, 1985). Antigenic variation of the causative organism, which makes it impossible to vaccinate against trypanosomiasis, constantly stimulates the host's immune system leading to excessive and often detrimental inflammatory reactions in the invaded tissues (Murray, 1979; Vickerman and Barry, 1982; Pentreath, 1989). Indeed, the lymphocyte/plasma cell infiltration and the astrogliosis of the central nervous system are responsible for most of the symptoms of late-stage sleeping sickness and most probably, its fatal outcome (Poltera, 1985).

Histological examination of brains from victims of reactive arsenical encephalopathy reveals a severe infiltration of the Virchow Robin spaces and the brain parenchyma by lymphocytes, plasma cells and macrophages; accompanied by diffuse reactive gliosis (Haller *et al*, 1986; Adams *et al*, 1986). Occasionally, an acute haemorrhagic encephalopathy, characterised by fibrinoid necrosis of the blood-vessel walls, possibly resulting from arsenic-induced vasculitis, has been reported in *gambiense* sleeping sickness (Adams *et al*, 1986). Many theories regarding the aetiology of this post-treatment encephalopathy (PTRE) have been proposed (reviewed by Hunter and Kennedy, 1992). The suggestion that PTRE is immune mediated has been widely supported (Galvao-Castro *et al*, 1978; Vickerman and Barry, 1982; Milord and Pepin, 1992). Consequently, corticosteroids have been used in conjunction with melarsoprol therapy in an attempt to prevent and/or reverse the development of the post-treatment encephalopathy. However, the results remain equivocal and the value of

corticosteroids in this respect is unclear (Foulkes, 1975; Arroz, 1987; Pepin *et al*, 1989a).

Eflornithine is a specific enzyme-activated, irreversible inhibitor of ornithine decarboxylase (ODC), the main regulatory enzyme for the *de novo* synthesis of polyamines. ODC synthesizes putrescine, the smallest polyamine, which is a precursor of spermine and spermidine (Seiler and Heby, 1988). Eflornithine was primarily used as an anti-cancer drug (Sunkara *et al*, 1982; 1983), but has also been used in chemotherapy of human African trypanosomiasis with some success (Van Nieuwenhove *et al*, 1985; Pepin *et al*, 1987; Doua *et al*, 1990). The immediate and dramatic effect of this trypanostatic drug on comatose sleeping sickness patients has raised the possibility that it may be acting in a capacity other than trypanostatic. Work performed, in our laboratory, on the chronic mouse model shows that it prevents the inflammatory cell infiltration into the CNS following subcurative trypanocidal therapy with Berenil even when the infecting *T.b. brucei* is eflornithine-resistant (J.M. Burke, personal communication). Similar observations have been made in a monkey model infected with an eflornithine-resistant *T.b. rhodesiense* stabilate, KETRI 2537. In this monkey model, the inflammatory cell infiltration, into the CNS, was observed to take longer to disappear when a curative melarsoprol regimen was used as compared to a non-curative eflornithine treatment (J.M. Ndung'u, personal communication). It, therefore, appears that eflornithine might be a promising supportive drug in the treatment of the late stages of African trypanosomiasis.

Since eflornithine is trypanostatic, it is possible that its beneficial effect on the trypanosome-induced CNS lesion might be a result of cure. For this reason, an eflornithine-resistant trypanosome stabilate was used in order to separate the trypanostatic effects of this drug, from the other potential effects on the inflammatory processes in the CNS of *T.b. brucei*-infected mice. In

addition, putrescine was used to reverse the eflornithine-induced, ODC-mediated inhibition of polyamine biosynthesis.

The correlation of astrocyte activation with cytokine production in *T.b. brucei*-infected mice (Hunter *et al*, 1991), and the observation during the course of this thesis (chapter 4) that cultured astrocytes exposed to trypanosomes and trypanosome-derived materials, produce cytokines implicates cytokines as the soluble mediators responsible for initiating the reactive gliosis and the inflammatory reaction observed in late-stage trypanosomiasis. Reactive gliosis may be beneficial when it walls off abscesses or detrimental when it interferes with CNS remyelination as in multiple sclerosis (Adams *et al*, 1983). By modulating the process of reactive gliosis it is conceivable that one may be able to enhance the astrocytic responses which promote functional recovery while blocking the detrimental effects associated with prolonged, extensive gliosis. It is possible that manipulating the production and the release of the cytokines by astrocytes may achieve such a modulation.

The aim of the present study is to determine how eflornithine might affect astrocyte activation, as judged by the intensity of GFAP staining and morphological changes *in vivo*, and by the production of cytokines *in vitro*.

## **5.2. MATERIALS AND METHODS**

### **5.2.1. The effect of eflornithine on the induction of post treatment reactive encephalitis**

A group of adult female NIH mice were infected with the eflornithine-resistant stabilate, GVR/35/C1.3 DFMO 5 (Chapter 2). Two percent eflornithine was administered *ad libitum* in drinking water for 7 or 14 days in a 2-hour light 4-hour dark environment to distribute the intake of eflornithine evenly (Gillet *et al*, 1986). Post-treatment reactive encephalitis (PTRE) was induced by treating the infected mice with Berenil on or after day 21 post infection (Chapter 2).

The infected mice were divided into six groups (Table 5.1). To investigate the optimal time for prevention of PTRE, one group of mice, E1, was treated with a single i.p. injection of Berenil on the 21<sup>st</sup> day of infection, at the same time as the 7 or 14 day oral administration of eflornithine was started. The second group, E2, was treated with eflornithine for 7 days, from day 21 after infection, prior to the treatment with Berenil, on day 28 after infection. Eflornithine therapy was continued for a further 7 or 14 days after this Berenil injection. Mice in groups E3 and E4, received eflornithine for 7 or 14 days, respectively, and an injection of Berenil at the end of the eflornithine course. Mice were sacrificed at the end of eflornithine course and every 7 days thereafter in groups of five.

Control mice consisted of: infected mice, treated with Berenil on either day 21 and 28 after infection (Group P1A and P1B), or with eflornithine from day 21 for 7 or 14 days (Group E0). Some uninfected mice were treated with Berenil or eflornithine or a combination of the two drugs as in the experimental groups. Mice which received Berenil alone were sacrificed every 7 days after the Berenil treatment, while the mice which received eflornithine were sacrificed on the last day of eflornithine therapy, and every 7 days, thereafter.

Some mice from the experimental groups and the infected control groups were retained and parasitaemia was monitored by examination of tail blood, twice every week.

### **5.2.2 The effect of eflornithine on an existing post treatment reactive encephalitis**

A group of female NIH mice were infected with the eflornithine-resistant *T.b.brucei* stabilate GVR/35/C1.3 DFMO 5. They were then divided into 2 groups (Table 5.2). The first group (E5) was treated with 40mg/kg Berenil i.p. on the 21<sup>st</sup> day of infection to induce a PTRE (Jennings *et al*, 1977) (Table 5.2).



**Table 5.2**  
**Treatment schedules with eflornithine during post-treatment reactive encephalitis**

P1	I	0	=====						K	49	52
			B	21	28	35	42	K	49	52	
			=====						K	49	52
P2	I	0	=====						K	63	70
			B	21	28	35	42	B	49	56	
			=====						K	63	70
E5	I	0	=====						K	49	56
			B	21	28	35	42	K	49	56	
			=====						K	49	56
E6	I	0	=====						K	70	77
			B	21	28	35	42	B	49	56	
			=====						K	70	77

The table illustrates the treatment schedules with eflornithine during an existing PTRE, in mice infected with the eflornithine-resistant trypanosome stabilate. Groups E5 and E6 were treated with eflornithine 7 days after the first (E5) and the second (E6) Berenil treatment. B = Berenil treatment, E = eflornithine treatment, K = killing time point. Numbers represent the time in days after infection. The information in this table has been provided in Chapter 2 but has been repeated, in a slightly different form, to facilitate the understanding of this Chapter.

Seven days after Berenil treatment, these mice received 2% eflornithine *ad libitum* in drinking water for 7 or 14 days. The second group (E6), received 40mg/kg Berenil on day 28 after infection and again after relapse of parasitaemia (which occurred 20-22 days after the first Berenil treatment), followed, 7 days later, by eflornithine for 7 or 14 days as in the first group. Five mice were, serially, sacrificed on the last day of eflornithine administration and 7 days (group E6) or 7 and 14 days (group E5), thereafter.

Control mice were infected and treated with Berenil alone on day 21 post infection, group P1, or on day 28 and again after relapse, group P2 (Table 5.2) . Serial killings were carried out as with the experimental animals. Uninfected control mice in groups of five were treated with either Berenil, eflornithine or a combination of the two as in the experimental groups. During the course of experiments, the mice were monitored for parasitaemia, by examination of tail blood.

### **5.2.3 The effect of simultaneous eflornithine and putrescine treatment on an existing post-treatment encephalitis**

A group of female NIH mice were infected with the eflornithine-resistant *T.b. brucei* stabilate GVR 35/C1.3 DFMO 5. The mice were treated with 40mg/kg Berenil on day 21 after infection and again after relapse of parasitaemia. Seven days after the second Berenil treatment, a group of these mice, EP, were simultaneously treated with 2% eflornithine in drinking water and 400mg/kg/day putrescine, i.p., for 14 days (Table 5.3). Mice from this group were sacrificed at the end of the eflornithine/putrescine course. The pathology in the brains of such mice was compared to that in the mice which were treated with eflornithine alone (Table 5.2, Group E6) after the two Berenil injections.

Control mice consisted of infected mice, (group PC) which were treated with Berenil on day 21 after infection and again after relapse of parasitaemia.



Seven days after the second Berenil administration, they were treated with 400mg/kg putrescine course and 7 days, thereafter. The pathology in the brains of this group of mice was compared to that in mice treated with eflornithine alone (group E6) after the two berenil treatments. Mice were monitored for parasitaemia every 2 days during the course of the experiment.

#### **5.2.4 Preparation of brains for histopathology**

At each kill, mice were perfused with approximately 150ml of normal saline, pH 7.0, through the left ventricle of the heart, to remove peripheral blood. The brains were removed, fixed in 5% buffered formalin and processed for histopathology. Three to 4 $\mu$ m transverse sections were cut serially through the fore-, mid-, and hindbrain. Immunocytochemistry for GFAP was performed on some of the sections to assess astrocyte activation, while the serial sections for all the samples were stained with haematoxylin and eosin for histopathology (Chapter 2).

#### **5.2.5 *In vitro* astrocyte preparation and exposure to eflornithine**

Cerebral cortices were dissected from 2 to 3-day old NIH mice and astrocyte preparation performed as described in Chapter 4. When purified and confluent they were divided into five groups of five flasks each. Lipopolysaccharide (LPS) was used as a known stimulant and compared to all other experimental stimuli. LPS at a concentration of 10 $\mu$ g/ml was used alone or in combination with eflornithine, while eflornithine was used at 0.5mM and at 5mM, alone and in combination with LPS. One flask in each group was not exposed to any stimulant and served as the baseline control. The cells were harvested by treating them with denaturing solution and detaching them with a rubber policeman, at 2, 4, 6 and 8 hours after exposure. They were then homogenised and stored at -70°C in phenol resistant plastic tubes. RNA extraction, cDNA preparation, amplification and detection of cytokine

transcripts were carried out as described in Chapter 4. The cytokines investigated included, IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF $\alpha$ , MIP-1 and GM-CSF as in Chapter 4.

### **5.3. RESULTS**

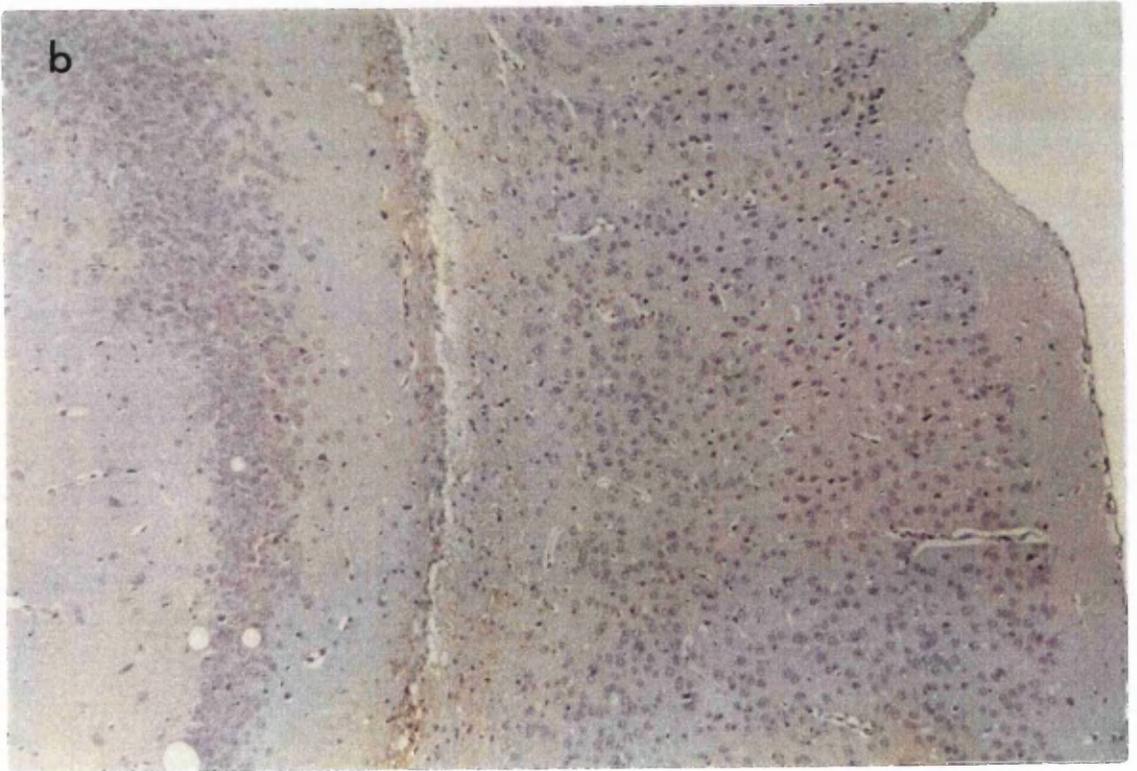
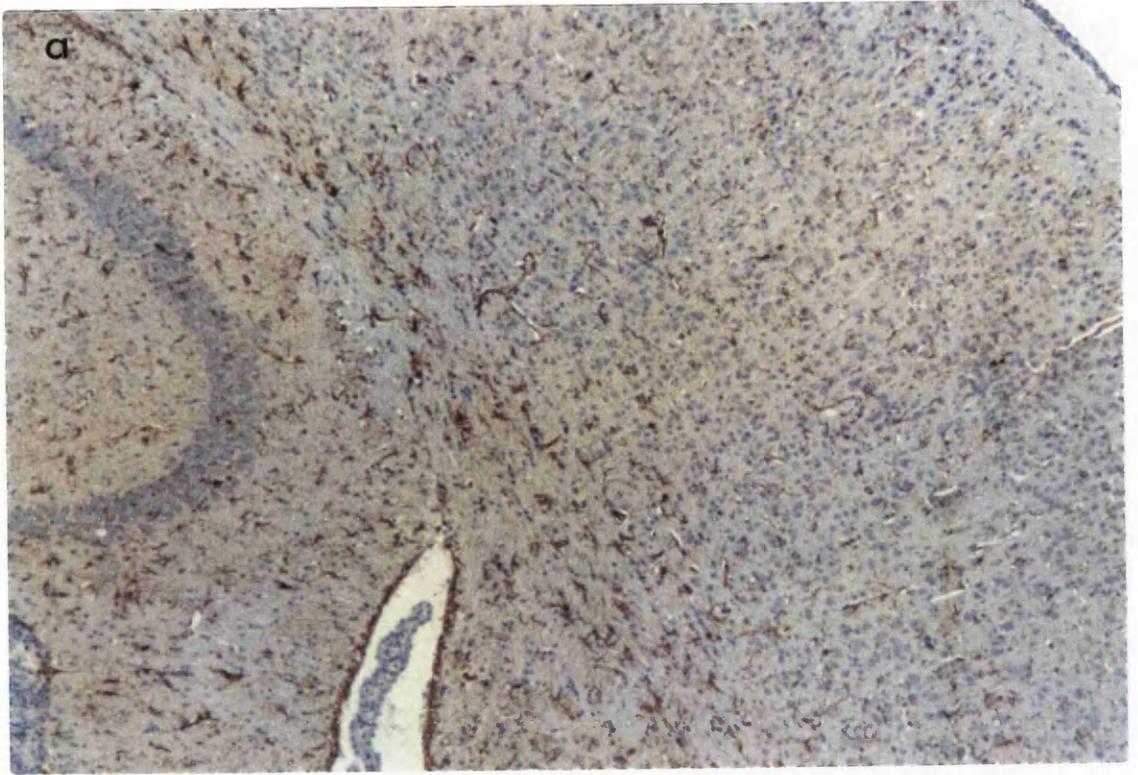
#### **5.3.1 The effect of eflornithine on the induction of post-treatment reactive encephalitis**

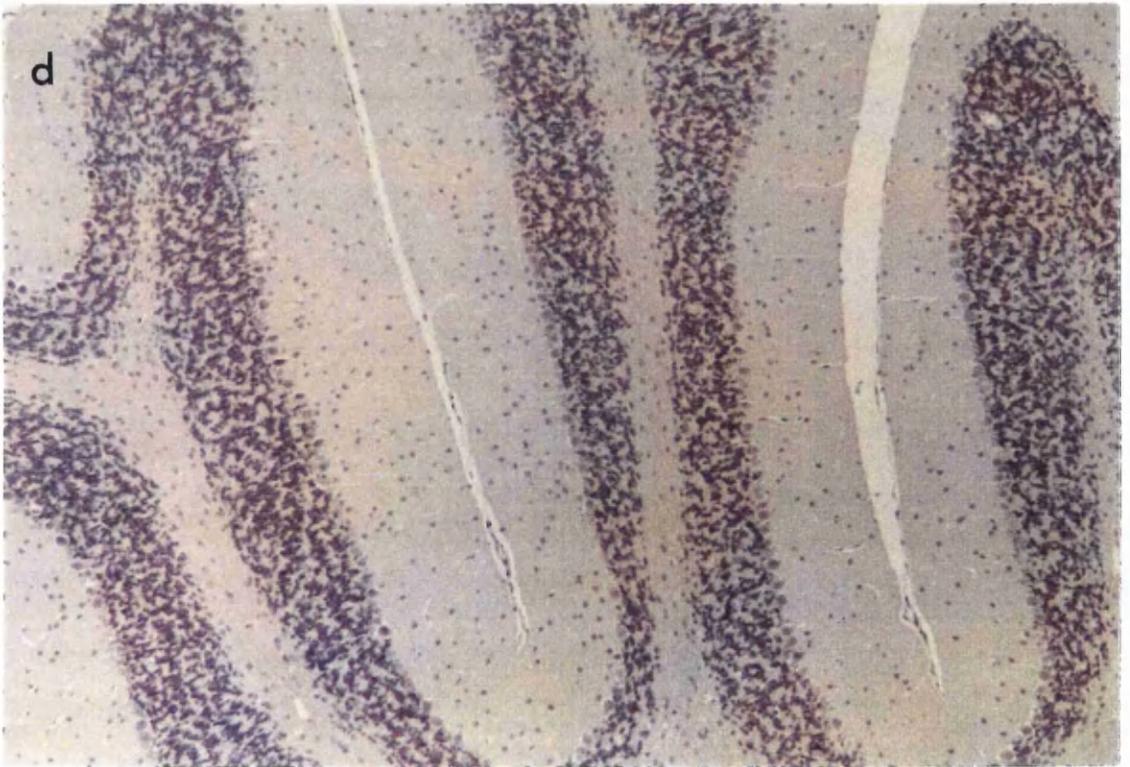
The pathology observed 7 days after induction of PTRE with Berenil (Group P1, Table 5.1) was: mild to moderate meningitis, perivascular cuffing and infiltration of the choroid fissure by inflammatory cells. The inflammatory cells were mainly lymphocytes and plasma cells, but there were a few macrophages as well. Perivascular cuffs were prominent in the cerebral white matter, along the hippocampal fissure, in the corpus callosum (Figure 5.1a), and in the cerebellar white matter, especially at the bases of the cerebellum (Figure 5.1c). Astrocyte activation, as judged by the intensity of GFAP staining, was marked along the inflamed meninges, around the perivascular cuffs and in areas of inflammatory-cell infiltration. Astrocyte activation appeared to diffuse from these areas of inflammation to the surrounding parenchyma with activation becoming less marked away from these inflammatory foci. Both the inflammation and astrocyte activation increased with time, with astrocytes becoming increasingly stellate and exhibiting more intense GFAP staining.

Mice that received eflornithine starting from the time of Berenil treatment (group E1) and sacrificed 7 days later, did not show any inflammation or perivascular cuffing (Figures 5.1b and 5.1d). In these mice, astrocyte activation was absent and histological sections were closely comparable to those from normal mice. This was also the case with the mice in group E2, which were given eflornithine for 7 days prior to injection with Berenil and continued receiving eflornithine for a further 7 or 14 days after the Berenil treatment.

### **Figure 5.1**

Comparison of pathology in cerebral cortices and the cerebellum of a mouse in group P1 sacrificed 7 days after induction of PTRE (a and c), compared to that in a mouse from group E1, which were treated with Berenil on day 21 and with eflornithine on days 21-28 after infection, and sacrificed at the end of eflornithine course (b and d). Note that eflornithine therapy prevented the development of inflammatory cell infiltrations and astrocyte activation. The sections were stained with rabbit anti-GFAP antibody, developed with a peroxidase/DAB kit (Vectastain) and counterstained with haematoxylin. x100





However, mild PTRE was observed in mice from groups E1 and E2, which were sacrificed 14 days after discontinuation of the eflornithine treatment. The severity of PTRE increased with the duration after discontinuation of eflornithine administration.

Mild PTRE was also observed in mice, from groups E3 and E4, which were treated with Berenil at the end of eflornithine course, and sacrificed 14 days after the Berenil treatment (Figures 5.2a and b). The severity of this PTRE increased with the duration after discontinuation of treatment.

Infected mice which were treated with eflornithine alone (group E0) and sacrificed at the end of eflornithine course, had neither astrocyte activation nor inflammatory cell infiltrates. Mild astrocyte activation and inflammatory cell infiltrations into the choroid fissure and the subarachnoid space, were found in mice which were kept alive for 14 days after discontinuation of eflornithine therapy. The inflammatory cell infiltrations and astrocyte activation, increased with time after discontinuation of therapy. Uninfected untreated mice and uninfected mice treated with either Berenil or eflornithine, or a combination of the two drugs did not develop any inflammatory cell infiltrations or astrocyte activation.

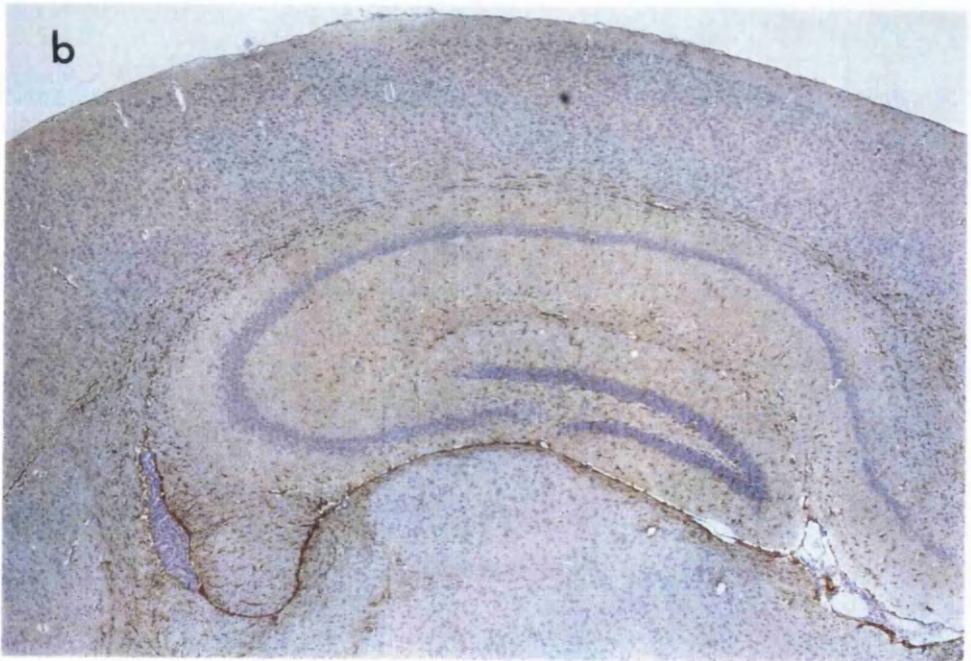
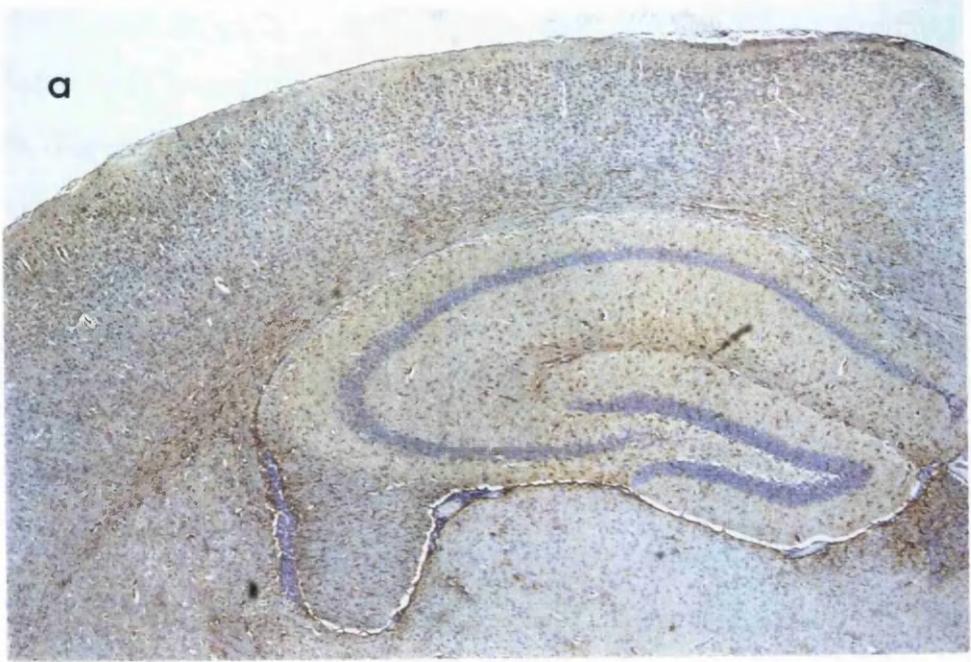
All the mice were parasitaemic at the end of eflornithine therapy, except those that received Berenil treatment at the end of eflornithine course, but which subsequently relapsed.

### **5.3.2 The effect of eflornithine on an existing post treatment reactive-encephalitis**

All the mice in this experiment were infected with the eflornithine-resistant trypanosome stabilate, in order to separate the trypanostatic effects of eflornithine from other effects it might have on the CNS pathology.

### **Figure 5.2**

Cerebral cortices comparing astrocyte activation in a mouse, in group E1, which received eflornithine from the day of PTRE induction and was sacrificed 14 days after discontinuation of eflornithine therapy (a) to that in a mouse, in the same group, which was sacrificed at the end of a 14-day eflornithine course (b). Note that inflammatory-cell infiltrations have recurred in the choroid fissure and in the meninges (a). Note also that astrocytes stain more intensely for GFAP and appear greater in number in mouse (a) than in mouse (b). The sections were stained with rabbit anti-GFAP antibody, developed with peroxidase/DAB kit (Vectastain) and counterstained with haematoxylin. x40

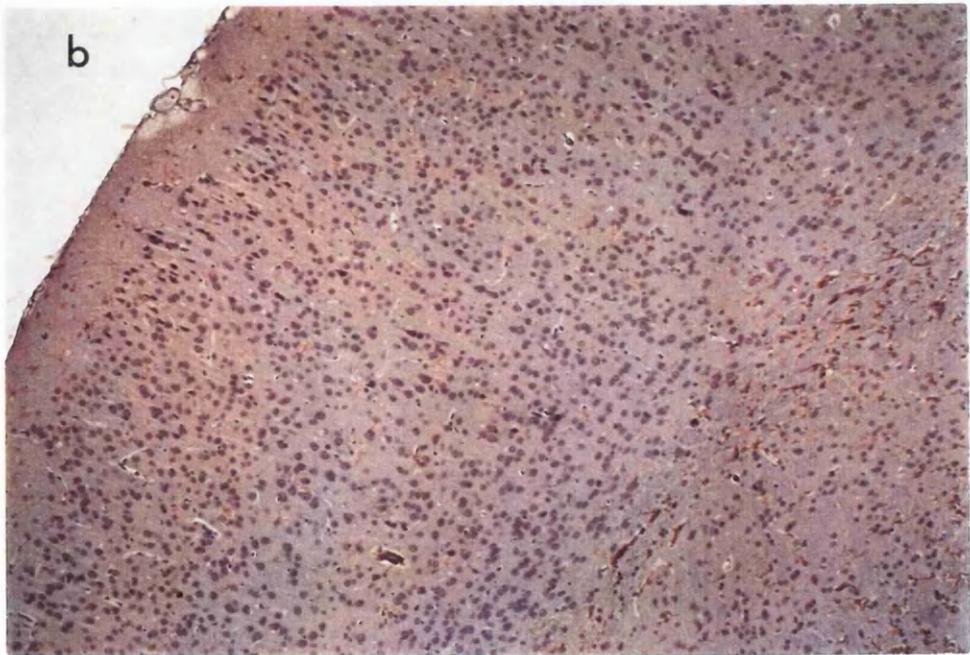
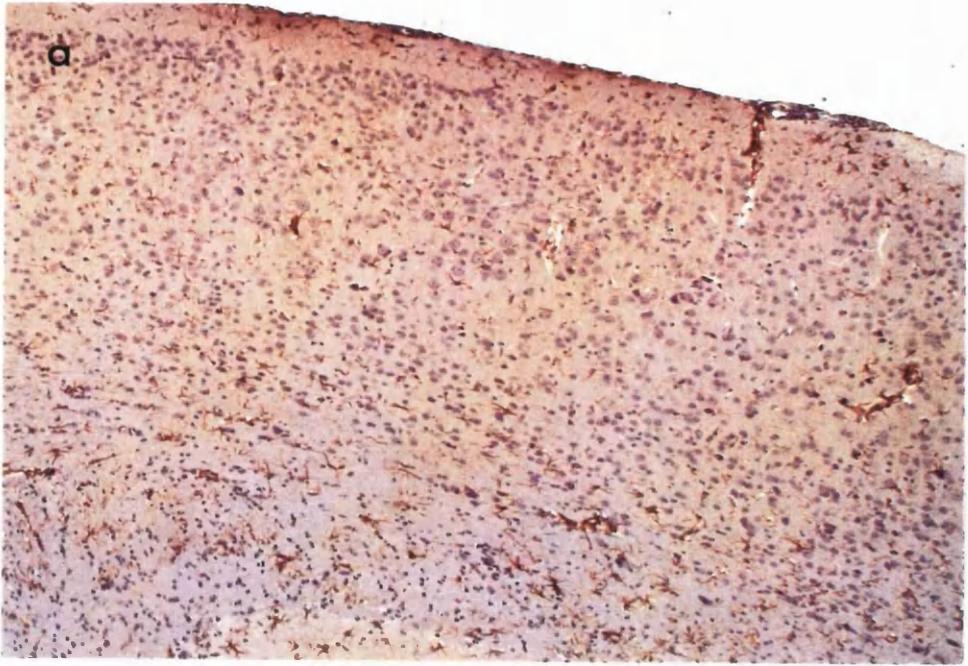


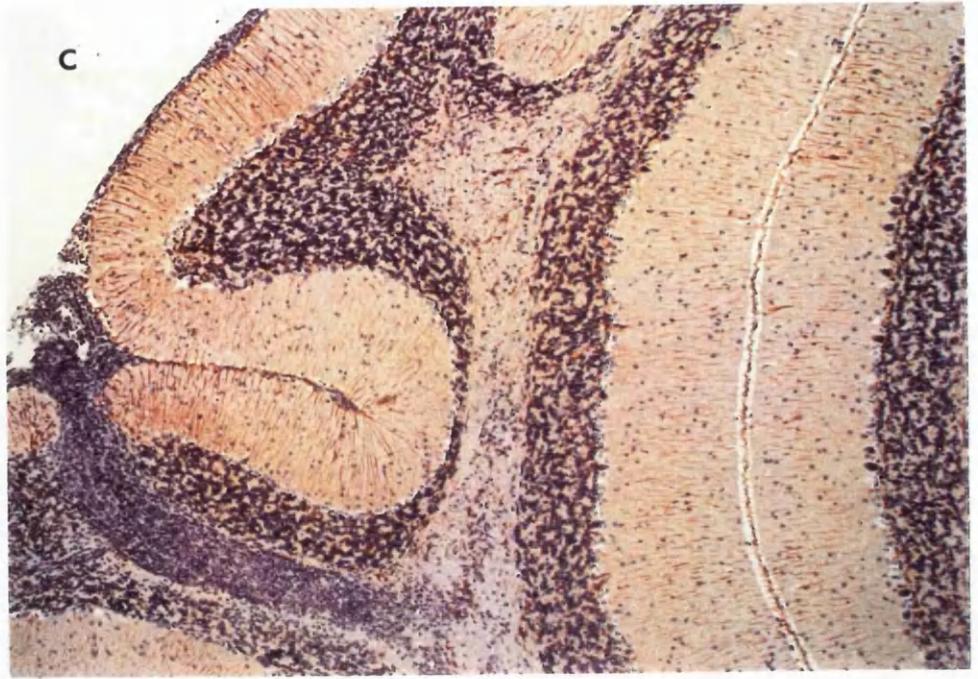
Mice from group P1, which were sacrificed 21 days after induction of PTRE had diffuse, moderately severe astrocyte activation and inflammatory cell infiltrates (Figure 5.3a and c). These figures were included here for the purpose of comparison with those from eflornithine-treated mice. Treatment with eflornithine for 14 days, starting 7 days after induction of PTRE (group E5), resulted in complete disappearance of inflammatory cells and a marked reduction in astrocyte activation in animals sacrificed at the end of the course of eflornithine (Figures 5.3b and d).

Histopathological sections of the brains from control animals that received two Berenil injections (group P2), exhibited a very severe PTRE consisting of meningitis (more than 10 cells deep), prominent perivascular cuffs diffusely distributed throughout the brain parenchyma, and infiltration of the cerebellar meningeal folds by numerous inflammatory cells. The cells consisted of macrophages, lymphocytes, plasma cells and Mott cells. In one of ten mice examined, neutrophils were present in all the inflammatory foci (Figures 5.4a and c). Activated astrocytes stained intensely for GFAP and exhibited elaborately branched cytoplasmic processes. The radial Bergmann glia stained for GFAP in the molecular layer of the cerebellum (Figure 5.4c). Mice that received eflornithine for 14 days from day 7 after the second Berenil treatment (group E6) and sacrificed at the end of eflornithine course, showed a remarkable reduction (almost complete resolution) in the degree of inflammation and astrocyte activation (Figure 5.4b and d). There was no meningitis although there were a few small perivascular cuffs and a few foci of inflammatory cells infiltrating the choroid fissure. The intensity of GFAP staining and the degree of astrocyte stellation was reduced by eflornithine therapy (Fig 5.4a and b). There was a similar reduction in inflammation and astrocyte activation in the cerebellum accompanied by a disappearance of radial GFAP staining of the Bergmann glia in the molecular layer (Figures 5.4c and d).

### **Figure 5.3**

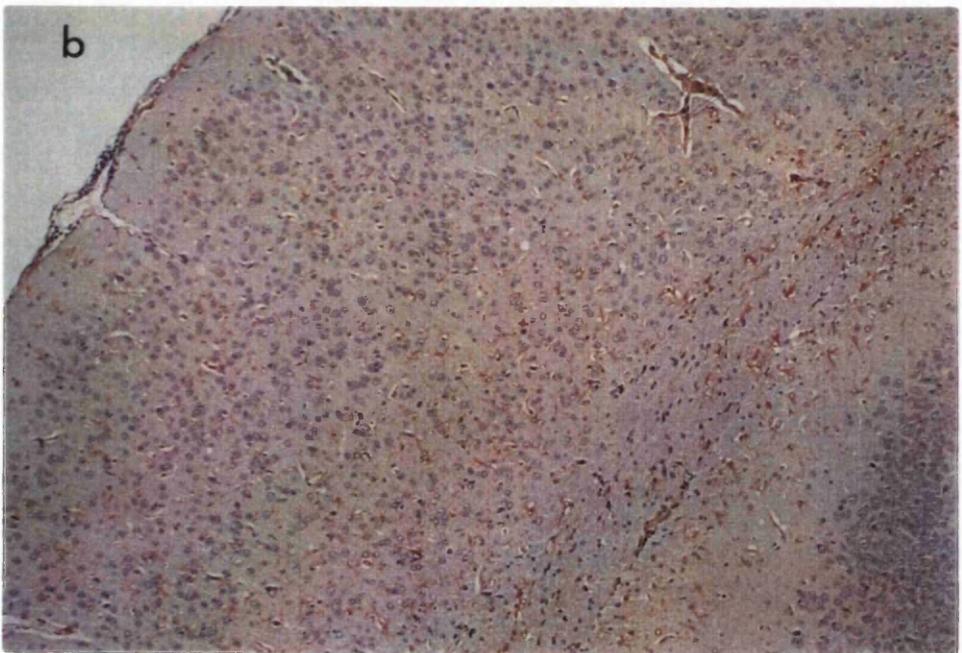
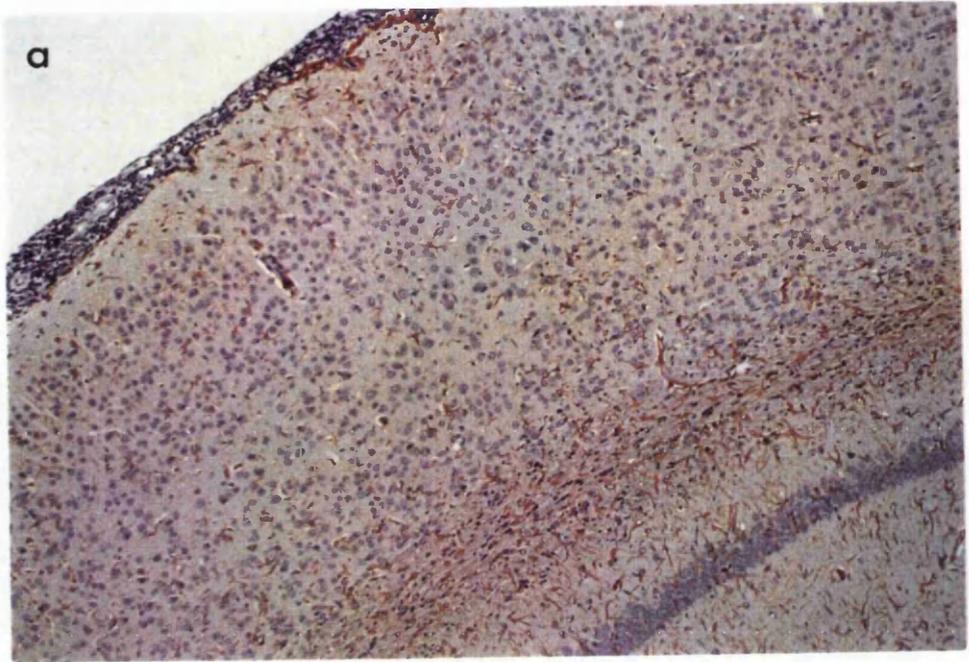
Comparison of astrocyte activation in a mouse from group P1, which was sacrificed 21 days after induction of PTRE (a and c), to that in a mouse, from group E5, which received eflornithine for 14 days, starting from 7 days after induction of PTRE and sacrificed at the end of eflornithine course (b and d). Eflornithine treatment completely reversed the inflammatory cell infiltration and resulted in a marked reduction in astrocyte activation. The sections were stained with rabbit anti-GFAP antibody developed with a peroxidase/DAB kit (Vectastain) and counterstained with haematoxylin. x90

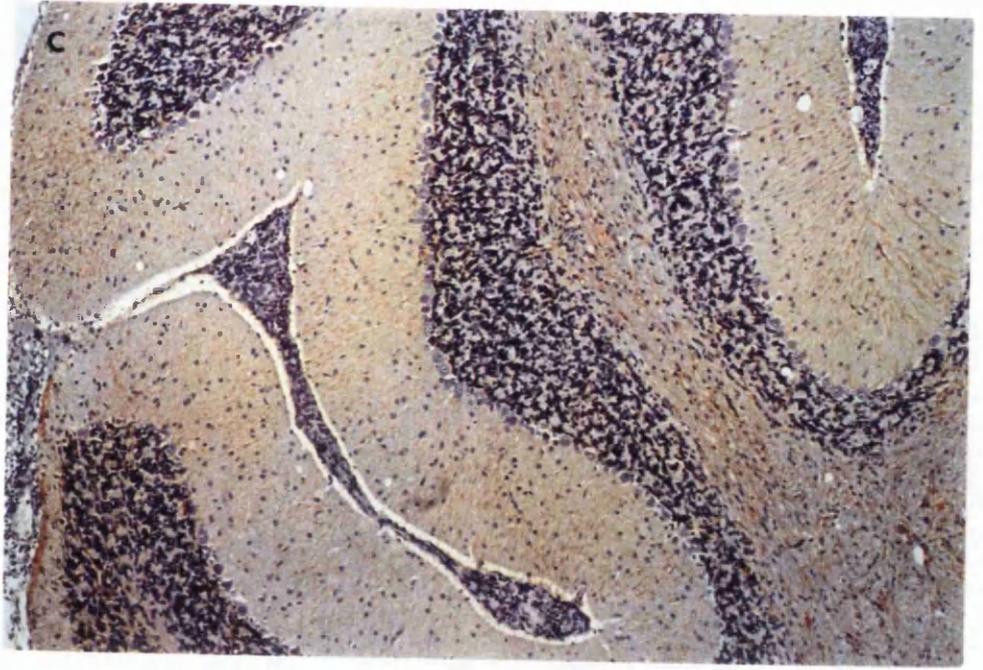




#### Figure 5.4

A comparison of pathology in the CNS of a mouse, from group P2, infected with the eflornithine-resistant stabilate of *T.b.brucei* GVR 35/C1.3 DFMO 5, treated with Berenil on day 21 after infection and again after relapse of parasitaemia, to that in a mouse, from group E6, that received eflornithine for 14 days, from day 7 after the second Berenil treatment. The mouse in Figure 5.4a and c was sacrificed 14 days after the second Berenil treatment while the one in Figure 5.4b and d was treated with eflornithine for 14 days starting 7 days after the second Berenil treatment, and sacrificed at the end of the eflornithine course. The photomicrographs show a great reduction in the inflammation and astrocyte activation in both the cerebral cortex (a and b) and the cerebellum (c and d). The sections were stained with rabbit anti-GFAP antibody developed with a peroxidase/DAB kit (Vectastain) and counterstained with haematoxylin. x90





Although post-treatment reaction was evident in mice sacrificed after 7 days of eflornithine therapy, the intensity of GFAP staining and the degree of astrocyte stellation were reduced.

### **5.3.3 The effect of simultaneous eflornithine and putrescine treatment on an existing post-treatment encephalitis**

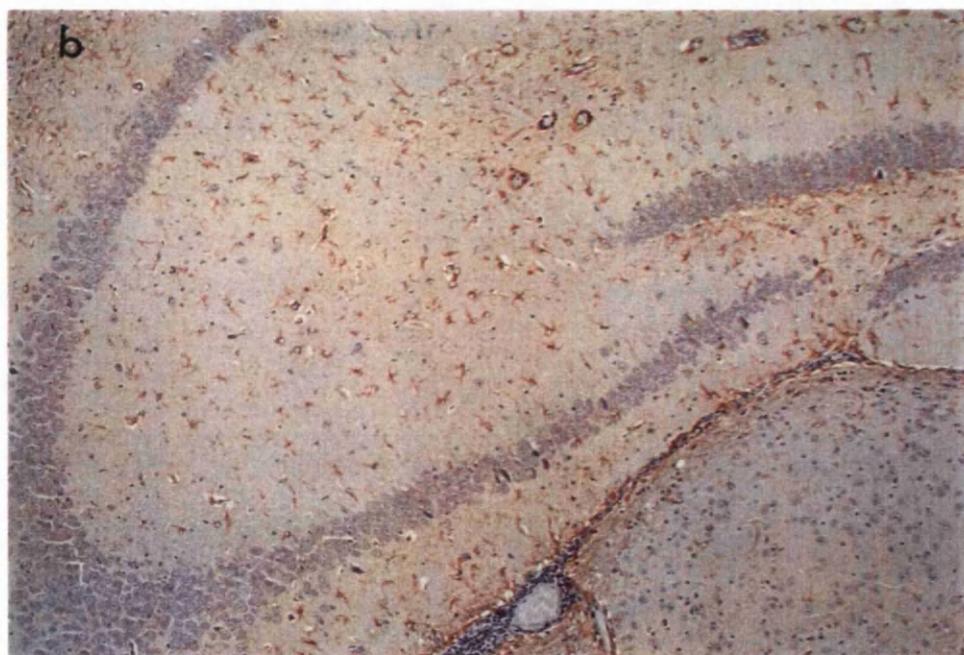
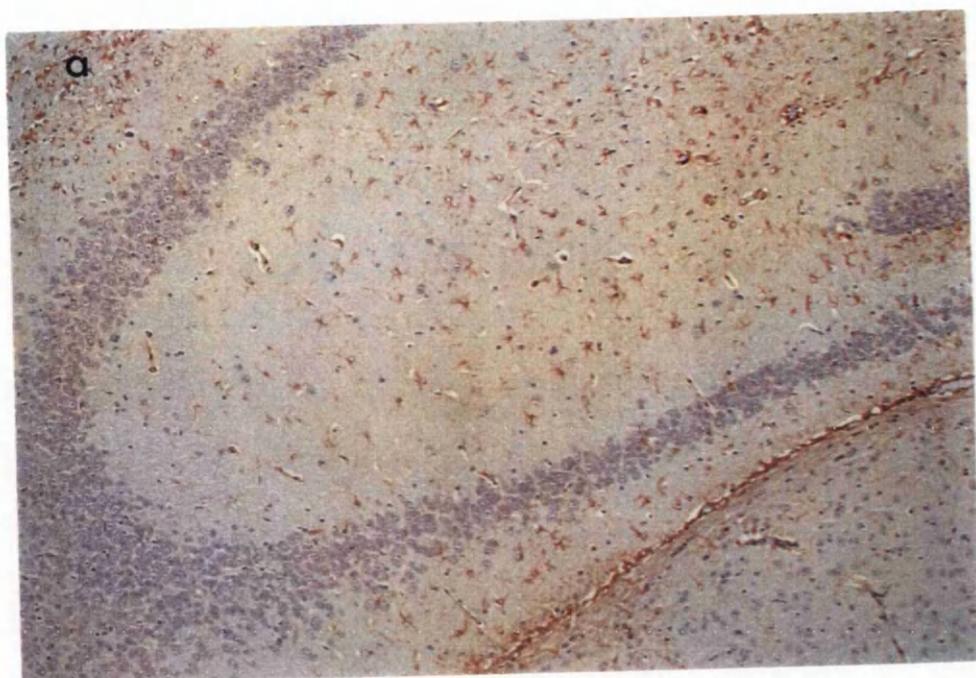
Mice, in group E6, which were treated with eflornithine alone for 14 days, after the double-Berenil treatment, did not show inflammatory cell infiltration into the perivascular spaces or the choroid fissure but there was mild meningitis in the cerebellum (Figure 5.5a and c). However, animals treated with eflornithine and putrescine simultaneously (group EP), after the double-Berenil regimen, developed astrocyte activation and inflammatory cell infiltrations (5.5b and 5.5d), but to a lesser degree than in the infected, Berenil-treated controls. The pathology in mice that received putrescine alone after the second Berenil treatment (group PC) was comparable to that seen in the Berenil-treated controls. All the mice were parasitaemic at the end of the putrescine therapy.

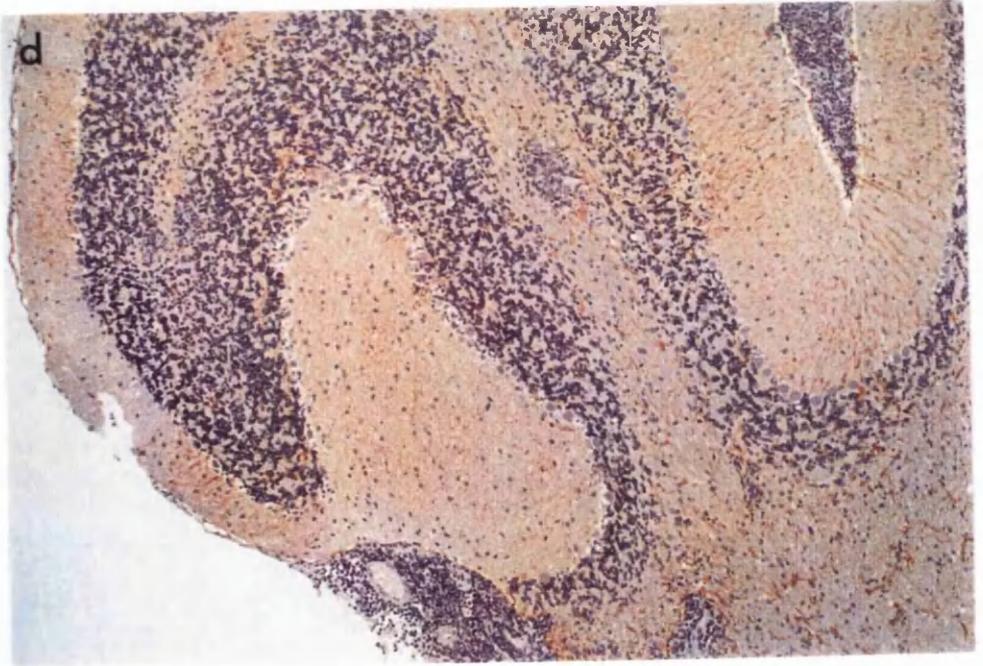
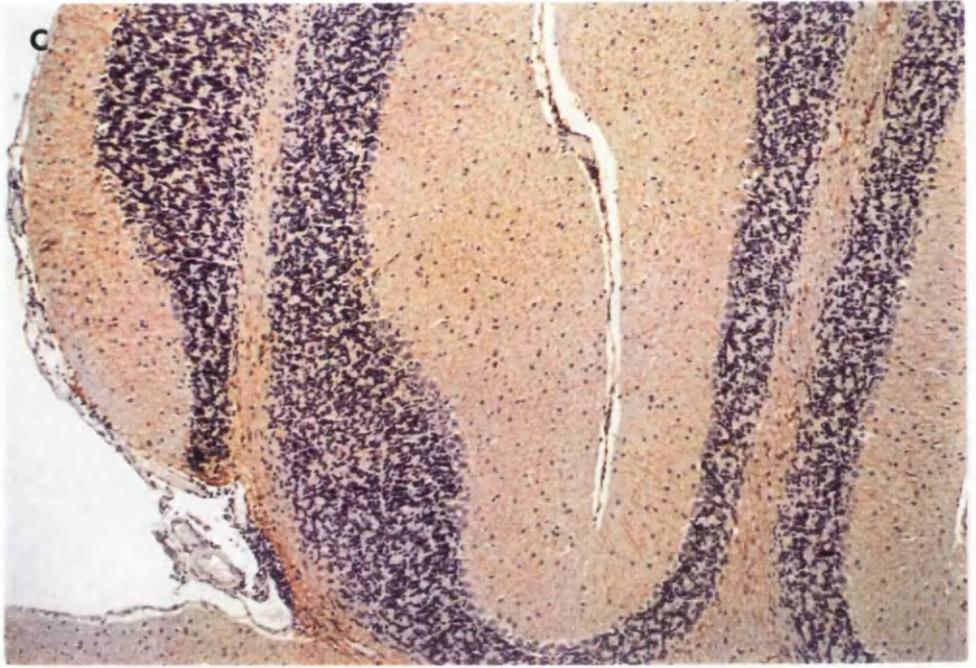
### **5.3.4 *In vitro* effects of eflornithine on astrocyte stimulation with lipopolysaccharide**

LPS at a concentration of 10 $\mu$ g/ml stimulated astrocytes to express mRNA transcripts for IL-1 $\alpha$  and - $\beta$ , IL-6, TNF $\alpha$ , MIP-1 and GM-CSF. Messenger RNA transcripts for all these cytokines were detectable by 2 hours after exposure to LPS. Although the cells used for this study are different from those used in Chapter 4, the variation of the individual cytokine transcripts were similar to those described in Chapter 4 and the results are presented Table 5.4. However, there are differences in the thickness of the bands. It is most likely that this was due to the differences sizes of the gel beds and combs. It is also possible that the different batches of cells produced different amounts of

### Figure 5.5

Brain sections comparing the pathology from a mouse in group Eo (a and c), that received eflornithine, to that in a mouse in group EP (b and d), that received a combination of eflornithine and putrescine, for 14 days starting 7 days after the second Berenil treatment. When eflornithine and putrescine were administered simultaneously, inflammatory cells infiltrated the choroid fissure and the perivascular spaces in the cerebrum (b), and the meningeal folds in the cerebellum (d). However, the degree of astrocyte activation in these two groups of mice appear very similar. The sections were stained with rabbit anti-GFAP antibody developed with a peroxidase/DAB kit (Vectastain) and counterstained with haematoxylin. x90





**Table 5.4**  
**Cytokine transcripts expressed by astrocytes following stimulation with lipopolysaccharide**

Cytokine	dose $\mu\text{g/ml}$	Time in hour:				
		C	2	4	6	8
IL-1 $\alpha$	10	-	+	++	+++	++
IL-1 $\beta$	10	$\pm$	$\pm$	+	$\pm$	$\pm$
IL-6	10	-	+	++	+++	-
TNF $\alpha$	10	-	+	++	++	$\pm$
GM-CSF	10	-	+	+	++	+
MIP-1	10	-	+	++	++	+

The table shows results of the semiquantitative PCR judging from the thickness of the bands produced after electrophoresis on 2% agarose gel and staining with ethidium bromide. The numbers represent time in hours after addition of LPS.

C: Unexposed cells which provided the baseline levels of cytokine transcript;

-: No band

$\pm$ : Faint band

+: Distinctly visible band

++: Fairly thick band

+++ : Thick band

cytokine transcripts.

Astrocytes exposed to 0.5mM eflornithine expressed low levels of mRNA transcripts for MIP-1 from the 2<sup>nd</sup> hour, a transient increase in the expression of IL-1 $\beta$  detectable 2 hours after exposure, and transient expression of TNF $\alpha$  and IL-6 starting from the 2<sup>nd</sup> and 4<sup>th</sup> hours, respectively. However, at this concentration, eflornithine did not induce expression of IL-1 $\alpha$  and GM-CSF transcripts (Table 5.5). A tenfold increase in eflornithine concentration caused a transient low level expression of MIP-1 transcripts and a reduction in IL-1 $\beta$  transcript to baseline levels, but did not induce expression of IL-1 $\alpha$ , IL-6, TNF $\alpha$  or GM-CSF transcripts (Table 5.5 and Figures 5.7 to 5.8).

The *in vitro* effect of eflornithine on astrocyte-stimulation by lipopolysaccharide was examined using the two eflornithine concentrations (described above). The lower concentration of eflornithine (0.5mM) delayed the LPS-induced expression of IL-1 $\alpha$  by 6 hours from 2 to 8 hours, when low levels of transcripts were detected (Figure 5.6). Although mRNA transcripts for the other cytokines studied (IL-1 $\beta$ , IL-6, TNF $\alpha$ , MIP-1 and GM-CSF) were detectable from the 2<sup>nd</sup> hour of stimulation, the levels were lower than those observed with LPS alone (compare Tables 5.4 and 5.6). When the concentration of eflornithine was increased to 5.0mM, i.e, ten fold increase, induction of IL-6 transcripts was completely inhibited while IL-1 $\beta$  was reduced to baseline levels. Expression of low levels of TNF $\alpha$  transcripts was detected 6 and 8 hours, while very low levels of IL-1 $\alpha$  transcripts were detected after 8 hours of exposure. There was a transient induction of GM-CSF transcripts in the first 4 hours which had disappeared by the 6<sup>th</sup> hour after stimulation. Low levels of MIP-1 transcripts were detectable throughout the study period (Table 5.6 and Figures 5.7, 5.8 and 5.9).

**Table 5.5**

The effect of eflornithine on the expression of cytokine transcripts by astrocytes *in vitro*

Cytokine	Dose mM	Time in hours				
		C	2	4	6	8
IL-1 $\alpha$	0.5	-	-	-	-	-
	5.0	-	-	-	-	-
IL-1 $\beta$	0.5	±	+	±	±	±
	5.0	±	-	±	±	-
IL-6	0.5	-	-	±	+	±
	5.0	-	-	-	-	-
TNF $\alpha$	0.5	-	±	+	±	±
	5.0	-	-	-	-	-
GM-CSF	0.5	-	-	-	-	-
	5.0	-	-	-	-	-
MIP-1	0.5	-	±	+	+	+
	5.0	±	±	+	+	±

The table shows results of the semiquantitative PCR judging from the thickness of the bands produced after electrophoresis on 2% agarose gel and staining with ethidium bromide. The numbers represent time in hours after addition of eflornithine. C: Unexposed cells providing the baseline levels of cytokine transcript;

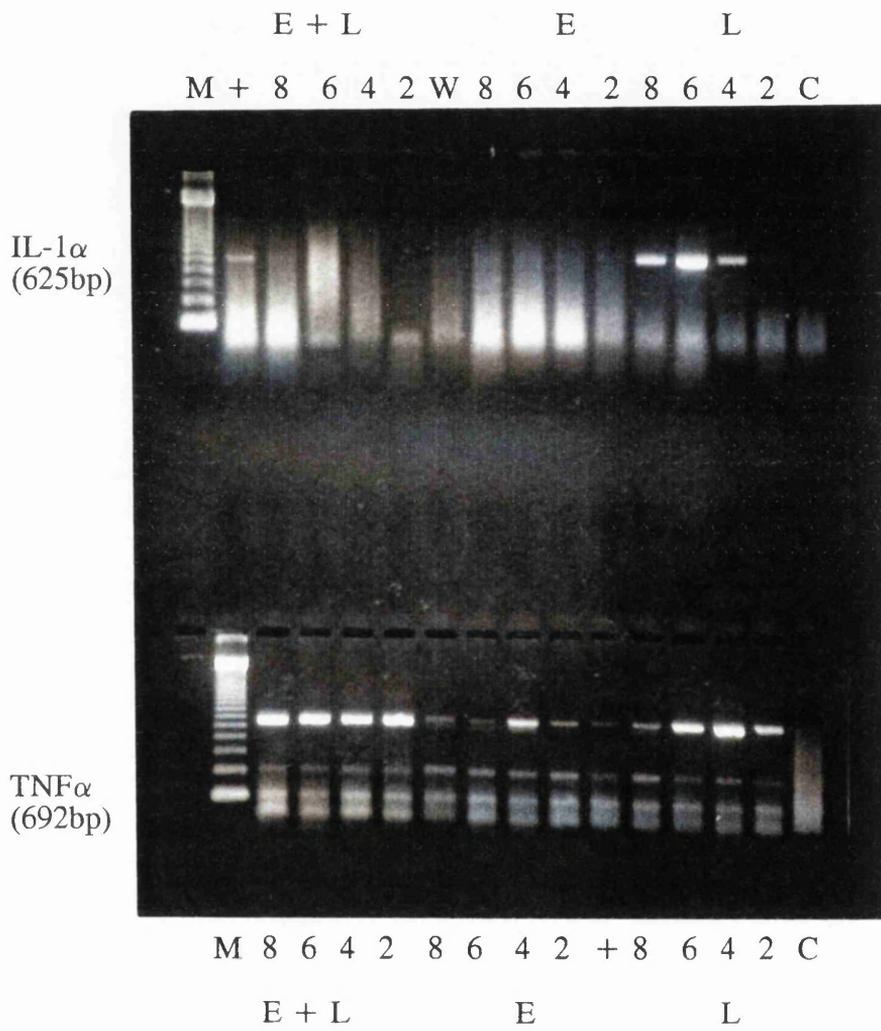
-: No band

±: Faint band

+: Distinctly visible band

### Figure 5.6

Gel electrophoresis showing the expression of mRNA transcripts for IL-1 $\alpha$  (top) and TNF $\alpha$  (bottom) in cultured astrocytes stimulated with 10 $\mu$ g/ml LPS (L), 0.5mM eflornithine (E) and a combination of LPS and eflornithine (E+L). This Figure shows that 0.5mM eflornithine delays the LPS-induced expression of IL-1 $\alpha$  transcript, while it has no effect on the expression of TNF $\alpha$ . The numbers, 2, 4, 6 and 8 represent the time in hours after stimulation. M = 123bp ladder (Gibco); + = the positive control; W = water, used as a negative control; C = unexposed cells.



**Table 5.6**  
**The effect of eflornithine on the expression of cytokine transcripts by Lipopolysaccharide-stimulated astrocytes *in vitro***

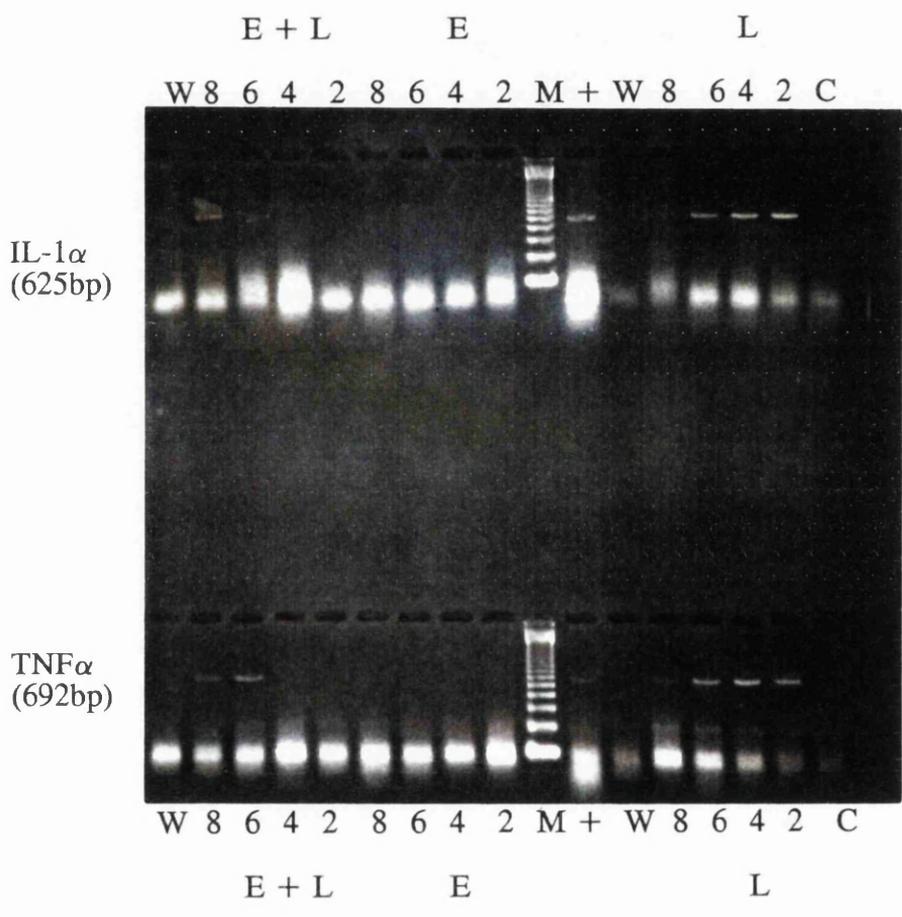
Cytokine	dose	Time in hours				
	mM	C	2	4	6	8
IL-1 $\alpha$	0.5	-	-	-	-	±
	5.0	-	-	-	-	+
IL-1 $\beta$	0.5	±	±	+	+	++
	5.0	±	±	±	±	±
IL-6	0.5	-	+	+	+	+
	5.0	-	-	-	-	-
TNF $\alpha$	0.5	-	+	+	+	+
	5.0	-	-	-	+	+
GM-CSF	0.5	-	+	+	+	+
	5.0	-	+	+	-	-
MIP-1	0.5	-	+	+	+	+
	5.0	±	+	+	±	+

The table shows results of the semiquantitative PCR judging from the thickness of the bands produced after electrophoresis on 2% agarose gel and staining with ethidium bromide. The numbers represent time in hours after addition of LPS and eflornithine. C: Unexposed cells which provided the baseline levels of cytokine transcript;

- : No band
- ±: Faint band
- +: Distinctly visible band
- ++: Fairly thick band

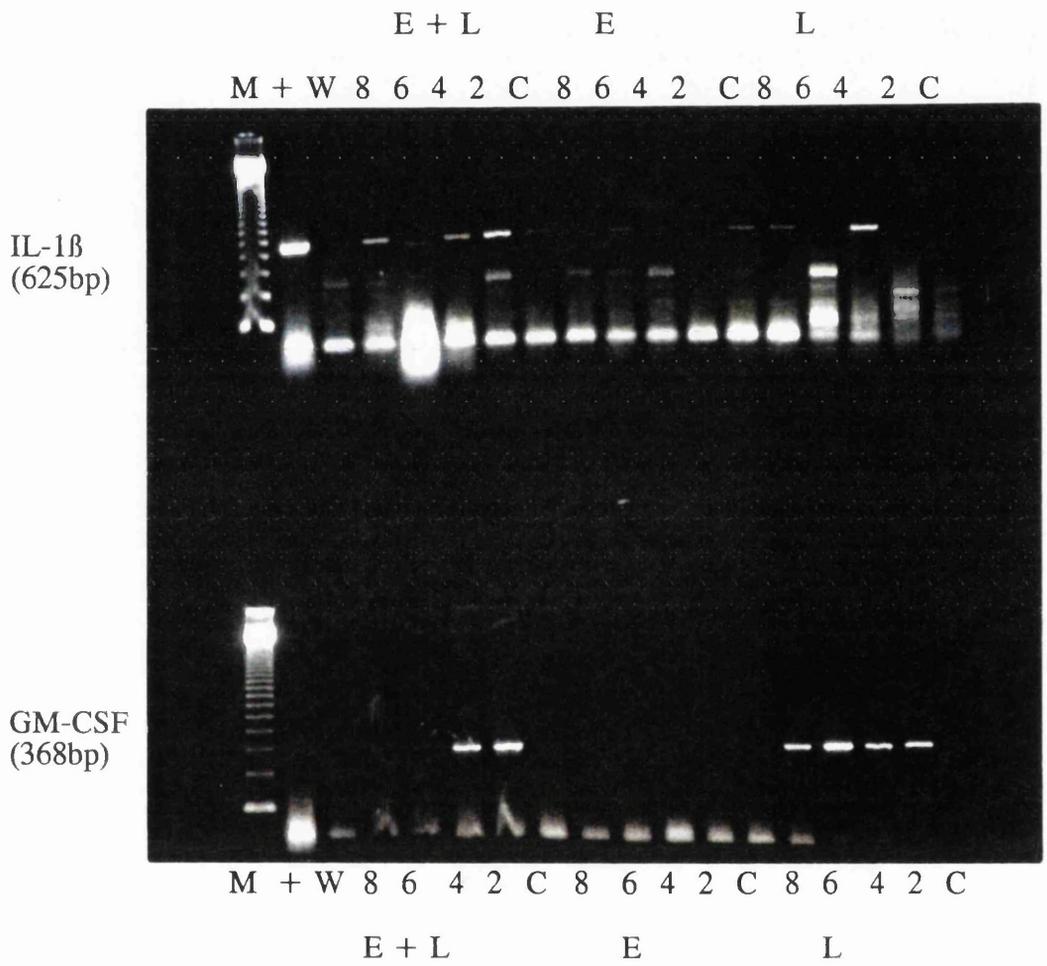
**Figure 5.7**

Gel electrophoresis showing transcripts for the cytokines, IL-1 $\alpha$  and TNF $\alpha$ , from cultured astrocytes exposed to 10 $\mu$ g/ml LPS (L) and 5.0mM eflornithine (E) and a combination of 10 $\mu$ g/ml LPS and 5.0mM eflornithine (E+L). The numbers, 2, 4, 6 and 8 indicate the time in hours after addition of stimulant; M = 123bp ladder (Gibco); + = positive control; W = water, used as a negative control; C = unexposed cells.



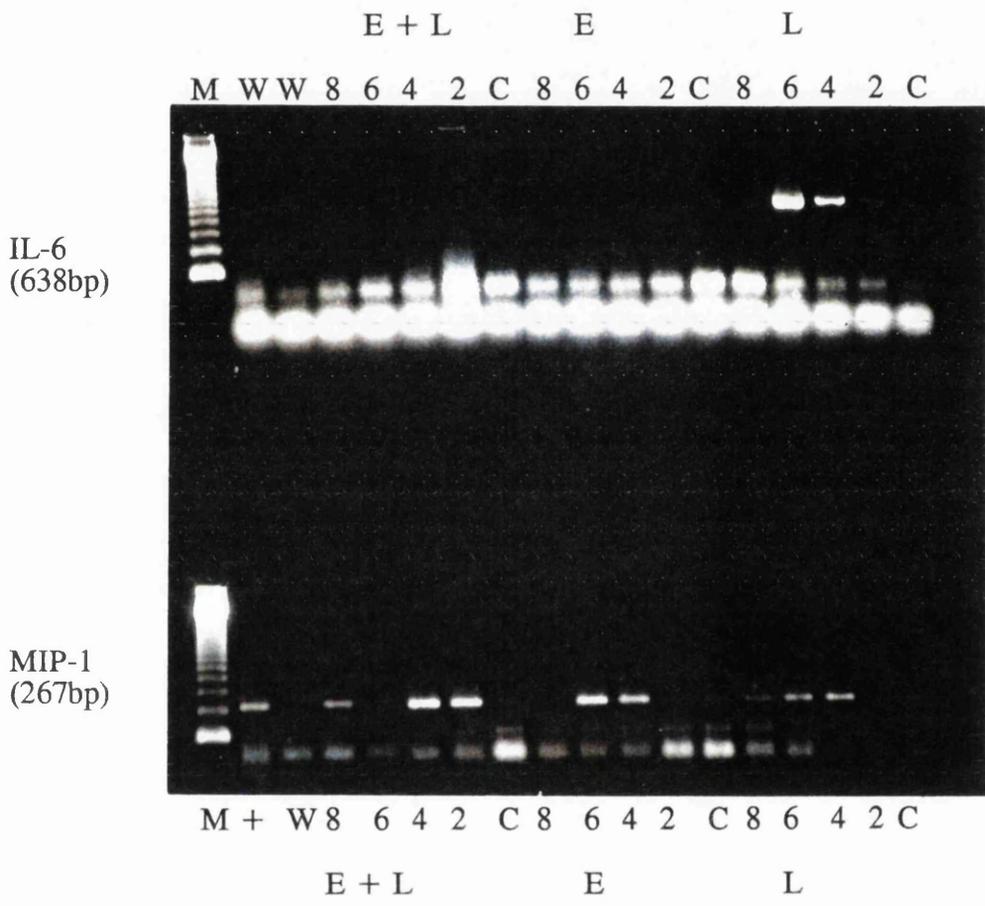
### Figure 5.8

Gel electrophoresis showing transcripts for the cytokines, IL-1 $\beta$  and GM-CSF, from cultured astrocytes exposed to 10 $\mu$ g/ml LPS (L) and 5.0mM eflornithine (E) and a combination of 10 $\mu$ g/ml LPS and 5.0mM eflornithine (E+L). The numbers, 2, 4, 6 and 8 indicate the time in hours after addition of stimulant; M = 123bp ladder (Gibco); + = positive control; W = water, used as a negative control; C = unexposed cells.



### Figure 5.9

Gel electrophoresis showing transcripts for the cytokines, IL-6 and MIP-1, from cultured astrocytes exposed to 10 $\mu$ g/ml LPS (L) and 5.0mM eflornithine (E) and a combination of 10 $\mu$ g/ml LPS and 5.0mM eflornithine (E+L). The numbers, 2, 4, 6 and 8 indicate the time in hours after addition of stimulant; M = 123bp ladder (Gibco); + = positive control; W = water, used as a negative control; C = unexposed cells.



#### 5.4. DISCUSSION

The results presented here showed that treatment with eflornithine prevents the development of PTRE and ameliorates an already established PTRE in animals infected with the eflornithine resistant *T.b. brucei* stabilate (GVR/35/C1.3 DFMO 5). The presence of circulating parasites was demonstrable throughout the course of eflornithine therapy until Berenil was administered. Since Berenil does not cross the blood-brain barrier in sufficient amounts to kill the trypanosomes in the brain parenchyma (Anosa *et al*, 1977; Jennings and Gray, 1983), the effect of eflornithine treatment on the CNS lesion observed here is not a result of successful cures. In addition, the observation that relapsed parasitaemia occurred in all animals that received a course of eflornithine around the first Berenil injection, indicates that the effect of eflornithine treatment on the CNS lesion, is independent of the trypanostatic effect of this drug.

The results of this study are the first to demonstrate that eflornithine inhibits the LPS-induced expression of cytokine transcripts by cultured astrocytes. Thus, eflornithine at 0.5mM inhibited the LPS-induced expression of cytokine transcripts for IL-1 $\alpha$  in cultured astrocytes. At higher concentrations (5.0mM), eflornithine inhibited expression of TNF $\alpha$ , IL-6 and GM-CSF, in addition to IL-1 $\alpha$ , and downregulated the expression of MIP-1 and IL-1 $\beta$ . Observations made by Hunter *et al* (1991) and also in the course of this thesis implicate astrocytes as the initiators of the CNS lesion seen in trypanosomiasis and in the post treatment reaction. The observation, in this study, that eflornithine inhibited the LPS-induced expression of cytokine transcripts in cultured astrocytes, indicates that eflornithine has a direct inhibitory effect on astrocytes. These results suggest that when eflornithine is used in late-stage sleeping sickness patients it acts in an anti-inflammatory as well as trypanostatic capacity, which would explain the dramatic reversal of clinical signs observed by various investigators (Van Nieuwenhove *et al*, 1985; Doua *et al*, 1990),

leading to eflornithine being described as the "the resurrection drug".

How the CNS inflammatory events are modified in the presence of eflornithine is not clear, but it seems unlikely that it compromises the immune system since its curative effect, as is evident in *T.b. gambiense* sleeping sickness, depends on an intact immune system (de Gee, McCann and Mansfield, 1983; Bitonti *et al*, 1986). This is further corroborated by the observations that the administration of 2% eflornithine in drinking water has no effect on the ability of normal mice to produce a haemagglutination response to horse red blood cells or oxazalone-sensitised (sensitised during eflornithine administration) mice to produce a delayed-type hypersensitivity when challenged with oxazalone (F.W. Jennings, unpublished data).

IL-1 seems to be a key factor in triggering a cascade of events that might lead to development of the CNS inflammatory lesion and its inhibition might be critical in modulating inflammatory reactions of the CNS. For instance, Merrill (1991) found that natural and recombinant forms of IL-1 and TNF $\alpha$  had a synergistic effect, amplifying microglial numbers four-fold over TNF $\alpha$  alone; however, anti IL-1 antibodies completely blocked this effect while anti-TNF $\alpha$  antibodies only partially did so. Furthermore, IL-1 or TNF $\alpha$  alone stimulated astrocytes to double in number and to triple when both cytokines were used together, in mixed glial cultures (Merrill, 1991). In addition, astrocytes underwent some putative differentiation that resulted in stellation when exposed to IL-1, but not TNF $\alpha$ , in only 3 days after addition of the cytokine. The effect on morphology was more dramatic when both cytokines were used together and was amplified by long term exposure (Merrill, 1991).

It has been proposed that some autocrine event involving IL-1, TNF $\alpha$  and a third factor, possibly IL-6 and/or platelet derived growth factor (PDGF), might work in tandem or in synergy to promote astroglia proliferation and differentiation (anti-IL-6 and anti-PDGF antibodies inhibit proliferation and

astroglial differentiation in mixed cultures) (Merrill, 1991). Both PDGF and IL-6 are some of the factors produced by astrocytes in response to IL-1 stimulation (Merrill, 1991). It is possible that PDGF and IL-6 are induced by and work in synergy with IL-1 and TNF $\alpha$  to produce proliferation and differentiation of astrocytes in late-stage trypanosomiasis. The results of this study show that exposure of cultured astrocytes to eflornithine, downregulates the LPS-induction of mRNA transcripts for IL-1 $\beta$ , IL-6, TNF $\alpha$ , MIP-1 and GM-CSF. It is possible that this occurs due to the lack of the autocrine activity of IL-1 $\alpha$  on astrocytes themselves, which may be needed to boost the levels of these cytokines to those observed with LPS alone. By inhibiting production of IL-1, eflornithine may be acting directly on astrocytes inhibiting their activation, thus downregulating the effect of this cytokine in the initiation and propagation of the CNS inflammation.

IL-1 increases expression of ICAM-1 and ELAM-1 on endothelial cells, altering the adhesive properties of the vascular walls, thus initiating leukocyte extravasation. It also induces the endothelial cells to produce chemotactic cytokines such as IL-8, macrophage chemotactic protein and colony stimulating factors which lead to leukocyte recruitment and proliferation (Broudy *et al*, 1987; Sieff *et al*, 1988; Montovani and Dejana, 1989). By preventing IL-1 production, eflornithine might curtail a cascade of events that could lead to the breakdown of the blood-brain barrier and could therefore prevent leukocyte extravasation and the resultant perivascular cuffs. IL-1 is a polyclonal activator of lymphocytes and it is possible that when eflornithine inhibits its production in the CNS by astrocytes, then the proliferative signal to the lymphocytes and plasma cells infiltrating the meninges and perivascular cuffs is removed, leading to the disappearance of these cells.

A tenable hypothesis is that diffusible paracrine factors derived from microglial cells or cell-surface molecules, trigger the changes seen in astrocytes in mixed glial cultures (Merrill, 1991). Indeed, a bi-directional effect might

occur with astrocytes triggering microglial cells to proliferate as well. Microglia do play a major role in gliosis *in vivo* and physically abut reactive astrocytes in brain tissue (Jordan and Thomas, 1988). They also produce factors *in vitro* such as glia-promoting factors (GPF) which stimulate astroglia proliferation (Giulian and Baker, 1985). In addition, activated mononuclear leukocytes and therefore presumably microglia, produce factors which stimulate astrocytic proteinases, a sign of astrocytic differentiation (Bever, Snyder, Endres, Morgan, Postlethwaite and Whitaker, 1989). This seems to suggest that the reduction in astrocyte activation following eflornithine therapy in the *T.b. brucei*-chronic mouse model studied here, is secondary to the disappearance of inflammatory cells. This is corroborated by the observation in this chapter that the inflammatory cells are the first to disappear followed by a reduction in astrocyte activation. However, the observation in the current study and earlier on (Chapter 3), that astrocyte activation disappeared faster when *T.b. brucei*-infected mice were treated with subcurative eflornithine regimen than when they were treated with the curative Mel Cy/MK436 combination regimen (Jennings, 1991c), suggested that eflornithine inactivates the astrocytes themselves.

Astrocytes produce both IL-3 and GM-CSF in response to IL-1 and TNF $\alpha$  both *in vivo* and *in vitro* (Nishida, Nikai, Kawakami, Aihara, Nishino and Hirari, 1989; Farrar, Vinocour and Hill, 1989). Although microglia do not proliferate in response to IL-1 *in vitro*, they multiply in response to IL-3 and GM-CSF (Frei *et al*, 1985; 1986; Giulian and Ingeman, 1988). It is possible that microglial hyperplasia contributes to the gliosis seen in late-stage trypanosomal infections and PTRE. Then, the inhibition of IL-1-induced, IL-3 and GM-CSF production by astrocytes, might explain how eflornithine downregulates the cerebral hypercellularity in the *T.b. brucei* chronic mouse model. It is possible that eflornithine inhibits production of IL-1 from cells other than astrocytes, including, lymphocytes, macrophages, endothelial cells and

microglia to produce the dramatic reduction in inflammation observed in this study.

Higher doses of eflornithine prevented the LPS-induction of mRNA transcripts for IL-1 $\beta$ , IL-6, TNF $\alpha$  and GM-CSF in addition to IL-1 $\alpha$ . The higher dose of eflornithine used in this study was 5mM eflornithine in culture medium. This dosage is over ten times the concentration of eflornithine expected to reach the CNS considering only 30% of plasma serum levels of eflornithine reach the CSF (Levin, Csejtey and Byrd, 1983; Bacchi and McCann, 1987). Since in a clinical intravenous regimen of 15g/day, plasma levels approach 1mM, it thus seems possible that peak levels of 100-300 $\mu$ M are attainable in the CSF (Van Nieuwenhove *et al*, 1985; Bacchi and McCann, 1987). This would be therapeutic since 50-100 $\mu$ M completely inhibits the growth of *T.b. brucei in vitro* (Giffin, McCann, Bitonti and Bacchi, 1986). While the 5mM eflornithine used in this study is higher than the levels of eflornithine expected to reach the CNS after oral administration, there is a chance that such high levels might reach the CNS following the disruption of the blood-brain barrier which seems to occur in late-stage sleeping sickness, especially after subcurative therapy (Waitumbi *et al*, 1988; Jennings *et al*, 1977). If the blood-brain barrier was disrupted and such levels of eflornithine were attained, it seems possible that eflornithine would be useful in downregulating the resultant inflammatory reaction that would occur in such cases.

Eflornithine competitively inhibits ornithine decarboxylase (ODC), an enzyme required for the biosynthesis of polyamines, which in turn are required by rapidly proliferating cells. It may therefore inhibit the proliferation of the astrocytes and the infiltrating inflammatory cells that form the CNS lesion in late-stage trypanosomiasis. Indeed it has been proposed that the inhibition of ODC by eflornithine is responsible for preventing proliferation of astrocytes during reactive gliosis (Zini, Zoli, Grimaldi, Melo-Pich, Biagini, Fuxe and Agnati, 1990). Blood-brain barrier disruption has been reported to evoke an

eflornithine-reversible activation of ODC (Koenig, Goldstone and Lu, 1989a; 1989b). Such a disruption might permit entry of factors and inflammatory cells that lead to reactive gliosis (Bologa, Cole, Chiappelli, Saneto and De Vellis, 1988; Giulian *et al*, 1988; Norton, Aquino, Hozumi, Chiu and Brosnan, 1992). These observations raise the possibility that conditions resulting in a compromised blood-barrier allow entry of blood-borne astrocyte growth factors/cell types that mediate an increase in GFAP through an ODC-dependent pathway (O'Callaghan and Seidler, 1992). The observation here that putrescine, a key polyamine synthesised by ODC, inhibited the eflornithine-induced amelioration of PTRE would support this hypothesis. Furthermore, the reduction in astrocyte activation occurred after the disappearance of the blood-derived inflammatory cells (macrophages, lymphocytes and plasma cells) and by inference the blood-derived factors. This would support the hypothesis raised earlier in this study, that astrocytes and infiltrating inflammatory cells are acting in co-operation to perpetuate the pathological lesion in late-stage trypanosomiasis and that this lesion is blood-brain barrier disruptive. It is possible that removal of inflammatory cells reduces the CNS cytokine levels to below the threshold required for diffuse astrocyte activation. The studies here show that eflornithine inhibits expression of transcripts for IL-1 $\alpha$ , IL-6, TNF and GM-CSF by LPS-stimulated astrocytes *in vitro*, indicating a direct suppressive effect of eflornithine on astrocytes. One way in which eflornithine might achieve this is by depleting polyamines which are required for RNA synthesis. Indeed, it has been reported that ODC, the enzyme inhibited by eflornithine, is a regulatory subunit of RNA polymerase 1 (Russell and Durrie, 1978) and that  $\beta$ -galactosidase mRNA elongation in *Escherichia coli*, is slowed by polyamine depletion (Morris and Hansen, 1973). The observation in this study that the PTRE observed when putrescine was administered with eflornithine, was not as severe as in the double-Berenil treated control, would

indicate that eflornithine is doing more than just inhibiting ODC.

The finding that eflornithine can prevent the occurrence of a severe post-treatment reactive encephalitis and by inference the "reactive arsenical encephalopathy", irrespective of its trypanostatic action, suggests that eflornithine supportive treatment in combination with melarsoprol would be an advantage even in *T.b. rhodesiense* infection in which it is not curative (Bacchi, *et al*, 1990). Post-mortem material from a *T.b. rhodesiense*-monkey model, in which eflornithine has been found to be completely refractory, showed that there was a modulation of CNS inflammation compared to materials from animals that died during the course of a curative melarsoprol regimen (J.M. Ndung'u, personal communication). Moreover, PTRE was reported to be absent in *T.b. brucei* infected-mice after treatment with a combination of low doses of eflornithine and melarsoprol (Jennings, 1988a; 1988b).

In conclusion, the results presented here suggest a dual role of eflornithine in preventing and ameliorating the meningoencephalitis found in late-stage trypanosomiasis: 1) In inhibiting the production of IL-1 by astrocytes, eflornithine prevents a cascade of events that would initiate and propagate the meningeal inflammatory process. Thus, it would inhibit the IL-1-induced migration of endothelial cells and expression of adhesion molecules on endothelial, as well as on glial cells, and therefore, the subsequent leukocyte extravasation and spread within the CNS. Eflornithine would also inhibit the IL-1-induced proliferation of infiltrating inflammatory cells. The downregulation of the IL-1-induced production of cytokines by the infiltrating inflammatory cells, the brain vascular endothelial cells, and the intrinsic glial and neuronal cells, would essentially remove the inflammatory boost provided by such cytokines. 2) By inhibiting ODC, eflornithine inhibits the synthesis of polyamines that would be required for the IL-1-induced, proliferation of inflammatory cells within the CNS.

The clinical implications of such effects cannot be overemphasised.

When used in conjunction with other trypanocides, eflornithine would prevent and/or attenuate post-treatment reactive encephalopathy, even when the infecting trypanosomes are eflornithine-insensitive. In addition, eflornithine is likely to be beneficial in the treatment of other CNS inflammatory conditions, such as viral and bacterial meningoencephalitis, that feature astrogliosis and inflammatory cell infiltrations.

## **CHAPTER 6**

### **THE EFFECT OF AZATHIOPRINE ON ASTROCYTE ACTIVATION *IN VIVO* AND *IN VITRO***

## 6.1 INTRODUCTION

The pathogenesis of the CNS disease in HAT, and of the reactive encephalopathy following treatment with melarsoprol is poorly understood (Adams *et al*, 1986; Haller *et al*, 1986). Evidence from experimental infections of laboratory and domestic animals, and from pathological lesions in the brains of HAT patients, implicate the host's immune response to the invading parasite as the underlying mechanism (Galvao-Castro *et al*, 1978; Greenwood and Whittle, 1980; Vickerman and Barry, 1982; Milord and Pepin, 1992; Cegielski and Durack, 1991). However, anti-inflammatory therapy with corticosteroids to prevent and/or attenuate reactive encephalopathy, yielded equivocal results and their value in that respect remains uncertain (Foulkes, 1975; Arroz, 1987; Pepin *et al*, 1989). Recently, the non-steroidal anti-inflammatory drug, azathioprine, has been shown to prevent and ameliorate the Berenil-induced infiltration of the CNS, by mononuclear inflammatory cells, in *T.b. brucei*-infected mice (Jennings *et al*, 1989; Hunter *et al*, 1992b).

Azathioprine is used in prevention of heterograph graft rejection (Elion, 1967; 1972). It has been reported to inhibit renal homograft rejections in dogs (Calne and Murray, 1961; Zukoski, 1965), prolong renal allograft in Rhesus monkeys (Dicke *et al*, 1971), and inhibits rejection of full thickness corneal xenograft in rabbits (Leibowitz and Elliott, 1966). It has also been shown to inhibit the secondary phase of adjuvant arthritis in a mouse model of arthritis (Whittington, 1970; Perper *et al*, 1971), and the development of experimental allergic encephalitis in rats, a model of multiple sclerosis (Ronsenthale *et al*, 1969; Babington and Wedeking, 1971). To date, azathioprine is still used in combination with other immunosuppressive drugs for prevention and prolongation of organ transplants in man (Isoniemi, Ahonen, Tikkanen, Van Willerbrand, Krogerus, Eklund, Hockerstedt, Salmela and Hayry, 1993; Vereerstraeten, 1993).

Since astrocytes have been implicated in the genesis of the CNS lesion

during trypanosome infections (Chapters 4 and 5), it is possible that modulating astrocyte activation might prevent the cascade of events that lead to the encephalitis and possibly the PTRE following subcurative trypanocidal therapy.

The aim of the present study was to determine how azathioprine, affects astrocyte activation, as judged by the intensity of GFAP staining and morphological changes, *in vivo*, and by the production of cytokines, *in vitro*.

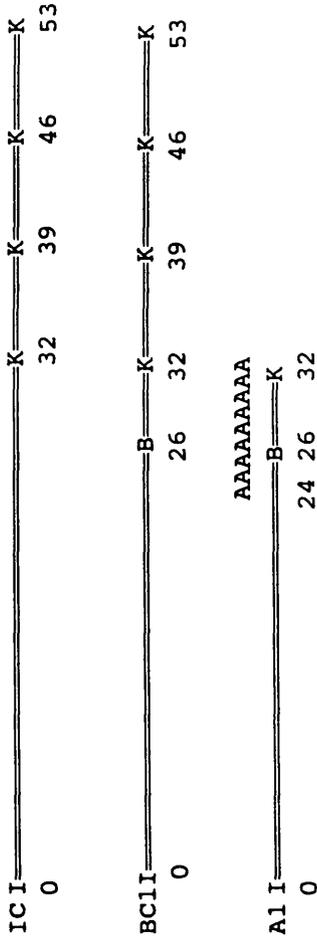
## **6.2 MATERIALS AND METHODS**

### **6.2.1 The effect of azathioprine on the induction of post-treatment reactive encephalitis**

Two groups of female NIH mice were infected with  $4 \times 10^4$  parasites of *T.b.brucei* GVR 35/C1.5 (Table 6.1). One group, BC1, was treated with 40mg/kg Berenil on day 26 after infection. Groups of five mice were sacrificed serially 6 days after this Berenil treatment and every 7 days thereafter. Another group of mice, A1, was infected and on days 24 to 32 after infection, they received 100mg/kg azathioprine and Berenil on day 26 after infection (2 days after the start of azathioprine administration). Groups of five mice were sacrificed serially on the last day of azathioprine therapy and every 7 days thereafter.

Control mice consisted of infected untreated mice (group IC), uninfected mice treated with either Berenil on day 26 or azathioprine on days 24 to 32, and uninfected mice treated with a combination of the drugs. Some mice from the experimental groups, and the infected control groups, were retained and monitored for parasitaemia.

**Table 6.1**  
**Treatment schedule for evaluation of the effect of azathioprine on the induction of post-treatment reactive encephalitis**



This illustrates the treatment regimen for evaluating the effect of azathioprine on the induction of PTRE in mice infected with the eflornithine-sensitive trypanosome stabilate. Group A1 received azathioprine from day 24 to day 32 of infection and a single Berenil treatment on day 26 after infection. The mice were compared with infected untreated controls (IC), and infected Berenil-treated mice (BC1). A = azathioprine, B = Berenil, I = Infection, K = killing time point. Numbers represent time in days after infection. The information in this table has been provided in Chapter 2 but has been repeated, in a slightly different form, to facilitate the understanding of this Chapter.

### **6.2.2 The effect of azathioprine on an existing post-treatment reactive encephalitis**

Two groups of female NIH mice infected with  $4 \times 10^4$  trypanosomes of the *T.b. brucei* stabilate GVR 35/C1.5, were treated with 40mg/kg Berenil on the 26<sup>th</sup> day of infection (Table 6.2). A group of these mice (A2) received 100mg/kg azathioprine i.p. for 7 or 14 days starting from day 32 after infection (7 days after the first Berenil injection), and sacrificed at the end of azathioprine course. The remainder, group A3, were treated again with 40mg/kg Berenil after relapse of parasitaemia, and with azathioprine, at 100mg/kg for 7 or 14 days from the day of the second Berenil treatment. Five mice were sacrificed at the end of azathioprine course, and the rest were sacrificed serially every 7 days thereafter in groups of five. Blood from tail snips was examined twice every week to monitor parasitaemia, throughout the study period.

These mice were compared to infected mice treated with Berenil alone, once (BC1) or twice (BC2), and sacrificed the same way as the experimental animals. Uninfected controls in groups of five were included for each drug regimen.

### **6.2.3 Preparation of brains for histopathology**

At each kill, mice were perfused with approximately 150ml of normal saline, pH 7.0, through the left ventricle of the heart to remove peripheral blood. The brains were removed, fixed in 5% buffered formalin and processed for histopathology. Three to 4 $\mu$ m transverse sections were cut serially through the fore-, mid-, and hindbrain. Immunocytochemistry for GFAP was performed on the sections to assess astrocyte activation. Serial sections from all the samples were stained with haematoxylin and eosin for histopathology (Chapter 2).



#### **6.2.4 *In vitro* astrocyte preparation and exposure to azathioprine**

Cerebral cortices were dissected from 2 to 3-day old NIH mice and astrocyte preparation performed as described in Chapter 4. When purified and confluent they were divided into four groups of five flasks each. Lipopolysaccharide (LPS) was used as a known stimulant and compared to all other experimental stimuli. LPS and azathioprine were used at a concentration of 10 $\mu$ g/ml alone or in combination with each other. One flask in each group was not exposed to any stimulant and served as the baseline control. The cells were harvested by treating them with denaturing solution and scraping them off with a cell scraper, at 2, 4, 6 and 8 hours after exposure. They were then homogenised and stored at -70°C in phenol-resistant plastic tubes. RNA extraction, cDNA preparation, amplification and detection of cytokine transcripts were carried out as described in Chapter 4. The cytokines investigated included, IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF $\alpha$ , MIP-1 and GM-CSF as in Chapter 4.

### **6.3 RESULTS**

#### **6.3.1 The effect of azathioprine on induction of post-treatment reactive encephalitis**

Mice in group BC1, that were sacrificed 6 days after induction of PTRE, exhibited a mild to moderate meningitis, perivascular cuffing and infiltration of choroid fissure by inflammatory cells, mainly lymphocytes and plasma cells, with a few macrophages. Perivascular cuffs, were prominent in the cerebral white matter, along the hippocampal fissure, in the corpus callosum, and in the cerebellar white matter, especially at the base of the cerebellum. Astrocyte activation, as judged by the intensity of GFAP staining, was marked along the inflamed meninges, around the perivascular cuffs and in areas of inflammatory-cell infiltration. Astrocyte activation appeared to diffuse

from these areas of inflammation to the surrounding parenchyma with activation becoming less marked away from these inflammatory foci. Both the inflammation and astrocyte activation increased with time, with astrocytes becoming increasingly stellate and exhibiting more intense GFAP staining.

Treatment of mice with azathioprine for 8 days starting from day 24 followed by a Berenil injection on day 26 after infection (group A1), prevented the infiltration of inflammatory cells into the CNS. However, astrocyte activation occurred along the meninges, around the blood vessels and the choroid plexus, and in the white matter in both the cerebrum and the cerebellum, although the extent of astrocyte activation was less than in the mice treated with Berenil alone, as assessed by the intensity of GFAP staining and the degree of astrocyte stellation (Figures 6.1a to f). However, the astrocyte activation at this time was greater than in infected untreated mice sacrificed on day 32 after infection.

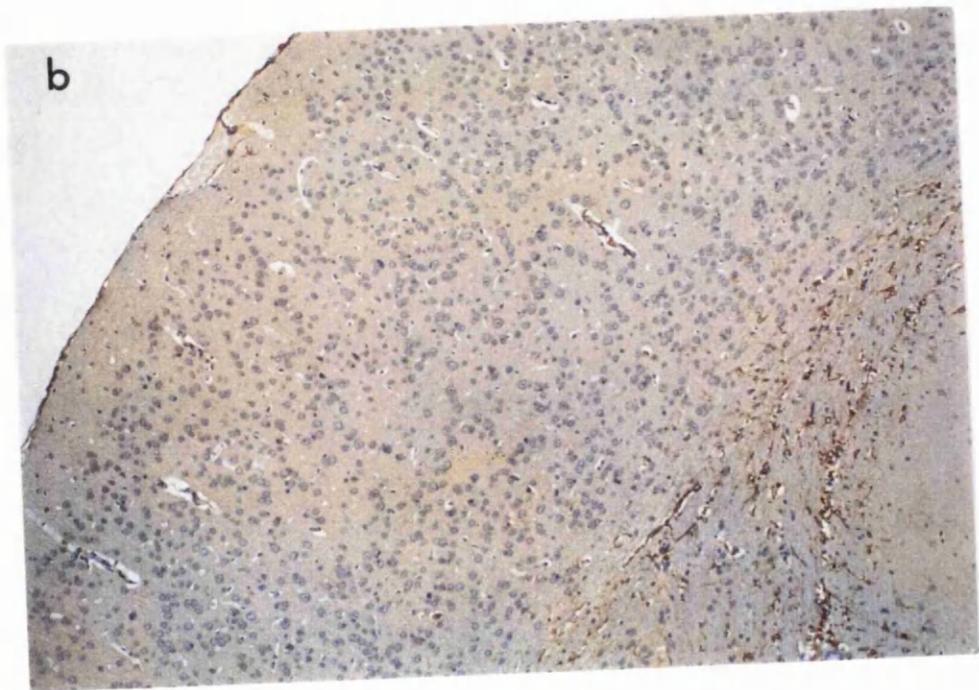
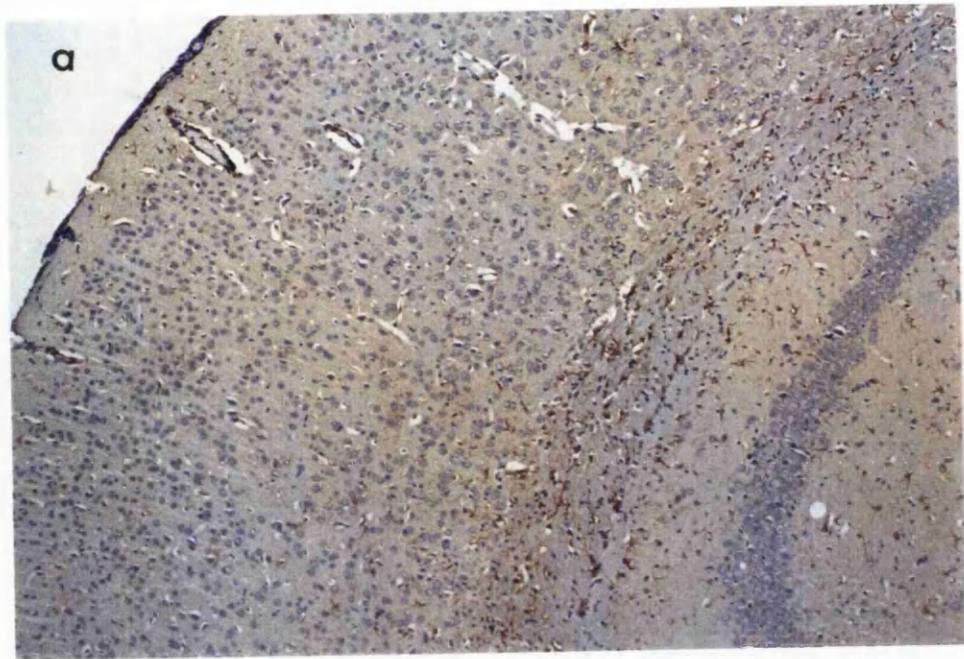
Uninfected mice, treated with either or both of the two drugs in this experiment did not develop CNS lesions. Blood from tail snips was examined twice every week for parasites, throughout the duration of experiment.

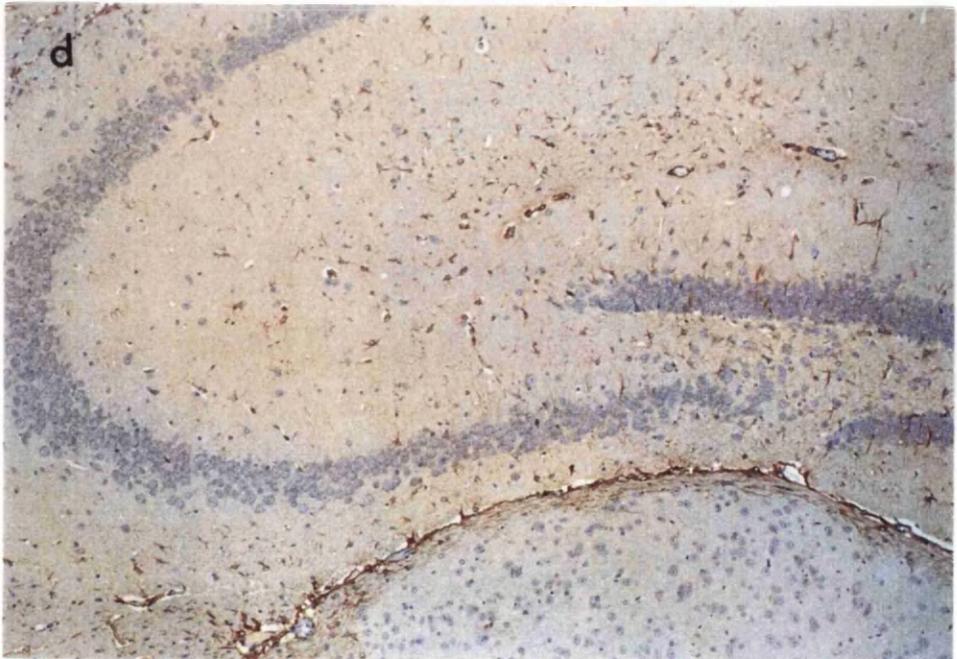
### **6.3.2 The effect of azathioprine on an existing post-treatment reactive encephalitis**

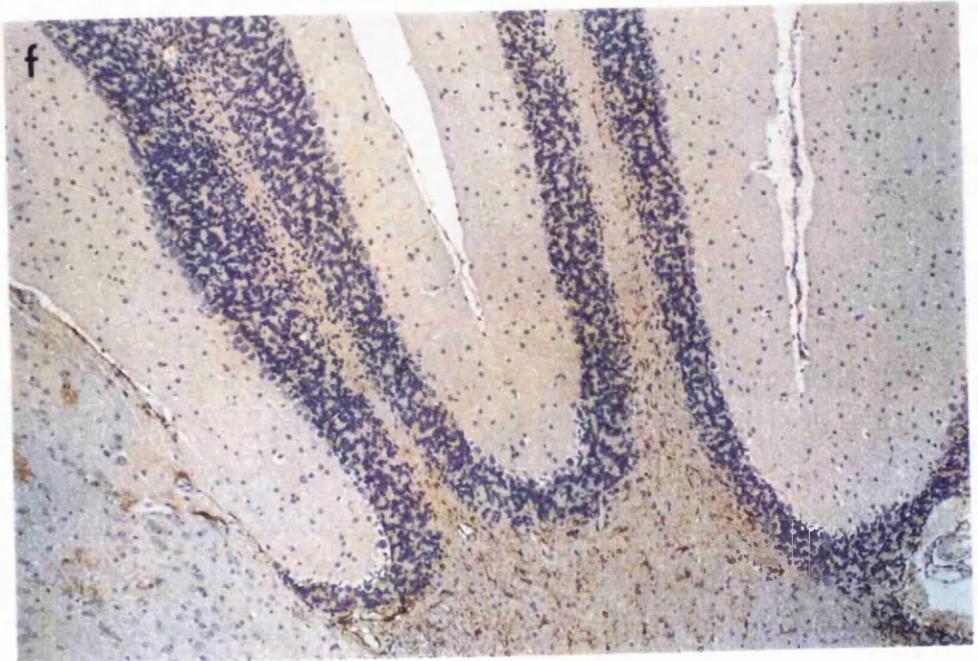
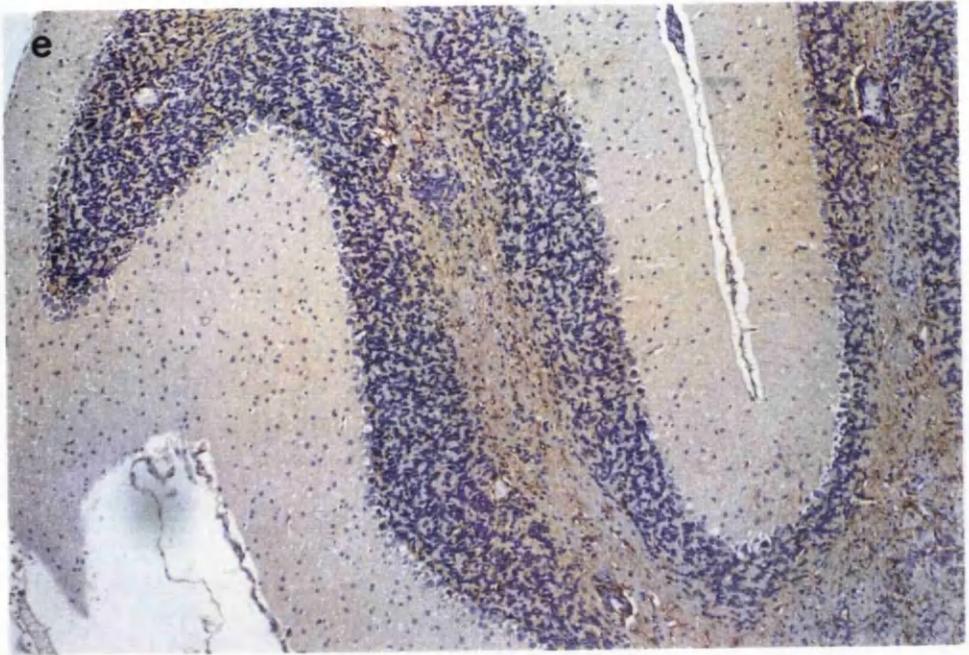
The group of mice that was treated with azathioprine for 7 or 14 days starting from 6 days after the Berenil treatment on day 26 after infection (group A2), developed focal, mild inflammatory cell infiltrations, and astrocyte activation along the meninges, the choroid fissure and the blood vessels. Although the astrocyte activation in this group of animals was less than in the Berenil-treated infected controls (group BC1), it was more marked than in mice that received azathioprine from two days before the Berenil injection (compare Figures 6.1 a,c and e with Figures 6.2a to c).

### Figure 6.1

Comparison of the CNS pathology and astrocyte activation in a mouse, from group BC1, infected with *T.b.brucei* GVR 35/C1.5 and treated with Berenil 26 days later and sacrificed 6 days after the Berenil therapy (a, c and e), to that in a mouse, from group A1, infected the same way, treated with Berenil on day 26 after infection and with azathioprine from the 24<sup>th</sup> to 32<sup>nd</sup> day of infection, and sacrificed at the end of azathioprine therapy (b, d and f). Azathioprine stops the meningitis and perivascular cuffing in the cerebral cortex (b); perivascular cuffing in the hippocampus and infiltration into the choroid fissure (d), and meningitis and perivascular cuffing in the cerebellum (f). Astrocytes stain more intensely, appear larger and are more diffusely distributed in animals that received Berenil alone. The sections were stained with rabbit anti-GFAP antibody, developed with peroxidase/DAB kit (Vectastain) and counterstained with haematoxylin. x90

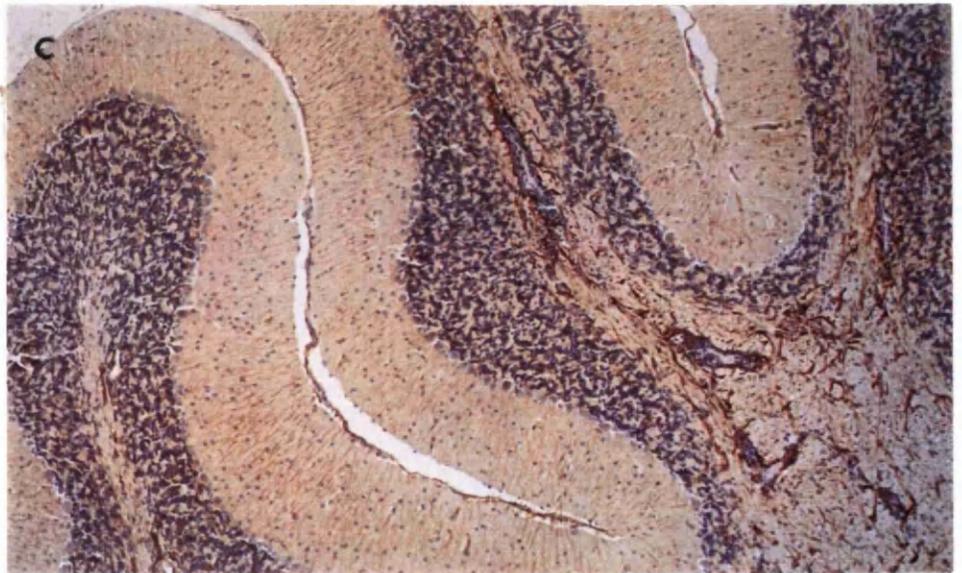
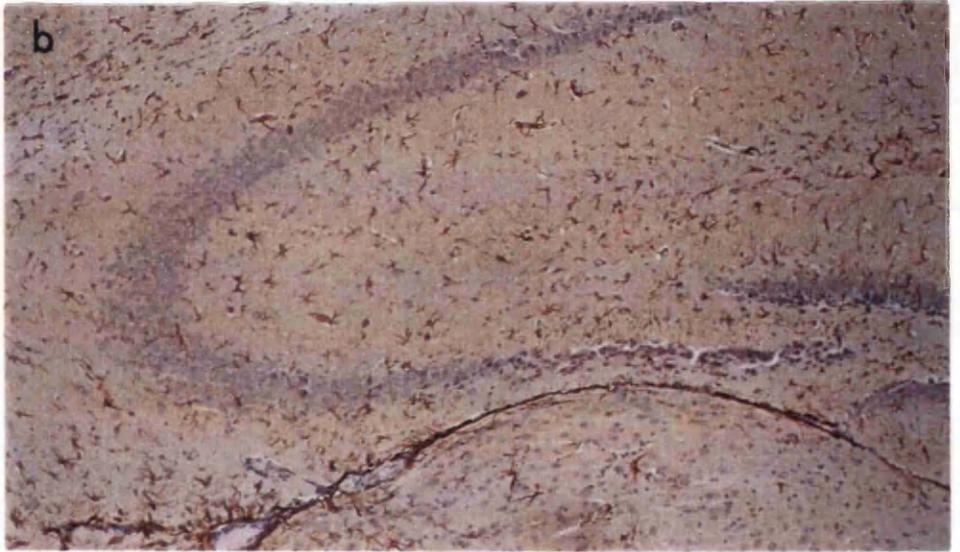
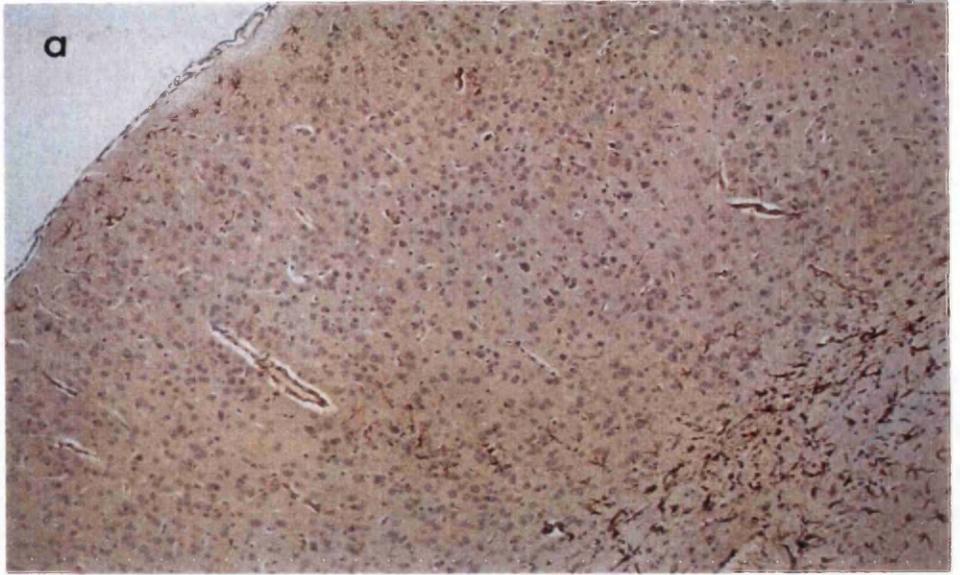






## Figure 6.2

Brain sections from a mouse in group A2, that was treated with Berenil on day 26 after infection with the eflornithine-sensitive trypanosome stabilate, followed by a 14-day azathioprine course. Azathioprine therapy was started on day 32 after infection (the 7<sup>th</sup> day after Berenil). While the inflammatory cell infiltrates in the choroid fissure (b) and the perivascular cuff in the cerebellar lobe (c) were not as severe as in mice treated with Berenil alone (Figures 6.1a, c and e), the astrocyte activation was comparable in the two groups (Figures 6.1a, c, e and 6.2 a,b, c). The sections were stained with rabbit anti-GFAP antibody developed with a peroxidase/DAB kit (Vectastain) and counterstained with haematoxylin. x90



The mice that received azathioprine for 7 days from the day of the second Berenil injection (group A3), showed a marked reduction in the meningitis and perivascular cuffing. However, azathioprine did not appear to affect the degree of astrocyte activation except in the cerebral grey matter where it was reduced. The number of astrocytes and degree of astrocyte stellation in the hippocampus and the cerebellum were comparable to those in the animals that received two Berenil treatments without azathioprine (group BC2). However, there was a slight decrease in the intensity of GFAP staining of the activated astrocytes (Figures 6.3a to d).

In this group of animals (A3), severe inflammatory-cell infiltration into the choroid fissure, the perivascular spaces and the meninges was evident 7 days after discontinuation of azathioprine administration.

### **6.3.3 *In vitro* effects of azathioprine on astrocyte activation**

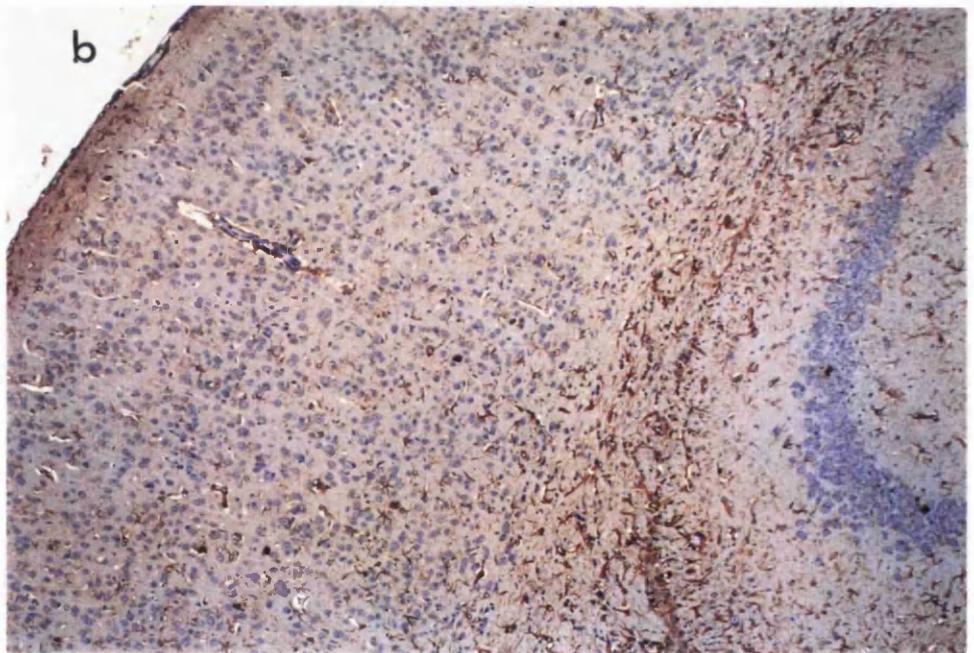
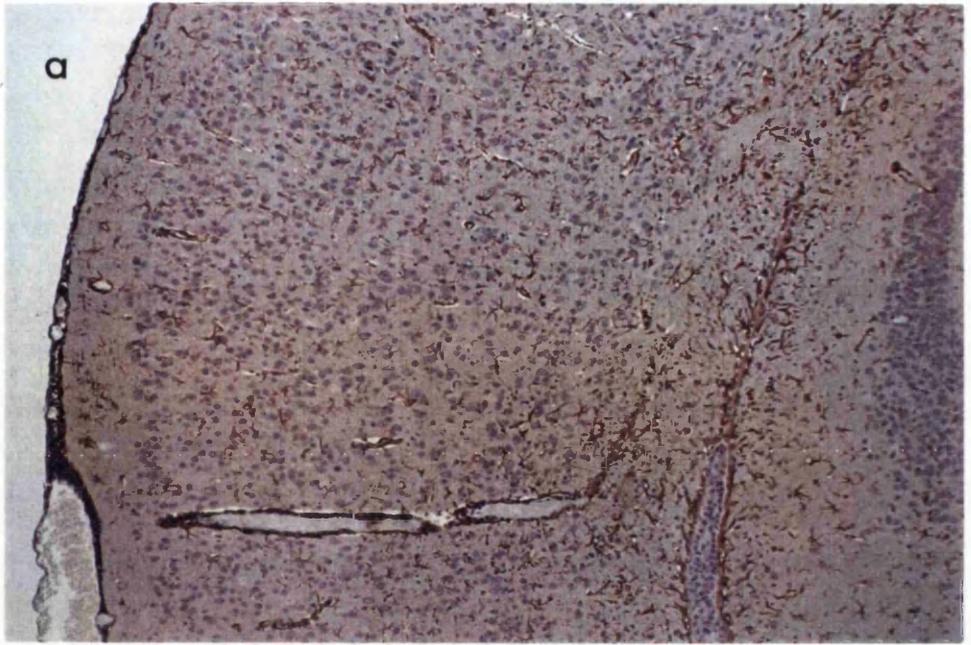
LPS at a concentration of 10 $\mu$ g/ml stimulated astrocytes to express mRNA transcripts for IL-1 $\alpha$  and - $\beta$ , IL-6, TNF $\alpha$ , MIP-1 and GM-CSF (Figures 6.4a to c). Messenger RNA transcripts for all these cytokines were detectable by 2 hours after exposure to LPS, with those of IL-6 and IL-1 $\beta$  reaching peak levels on the 4<sup>th</sup> and 6<sup>th</sup> respectively and remaining at this level for the rest of the experimental duration. Those of MIP-1 remained at the same level except for a slight decrease detected at the 4<sup>th</sup> hour. Transcripts for IL-1 $\alpha$  seem to have peaked at the sixth hour and were declining by the 8<sup>th</sup> hour, while those of GM-CSF had disappeared by the 8<sup>th</sup> hour after peaking between the 4<sup>th</sup> and the 6<sup>th</sup> hours.

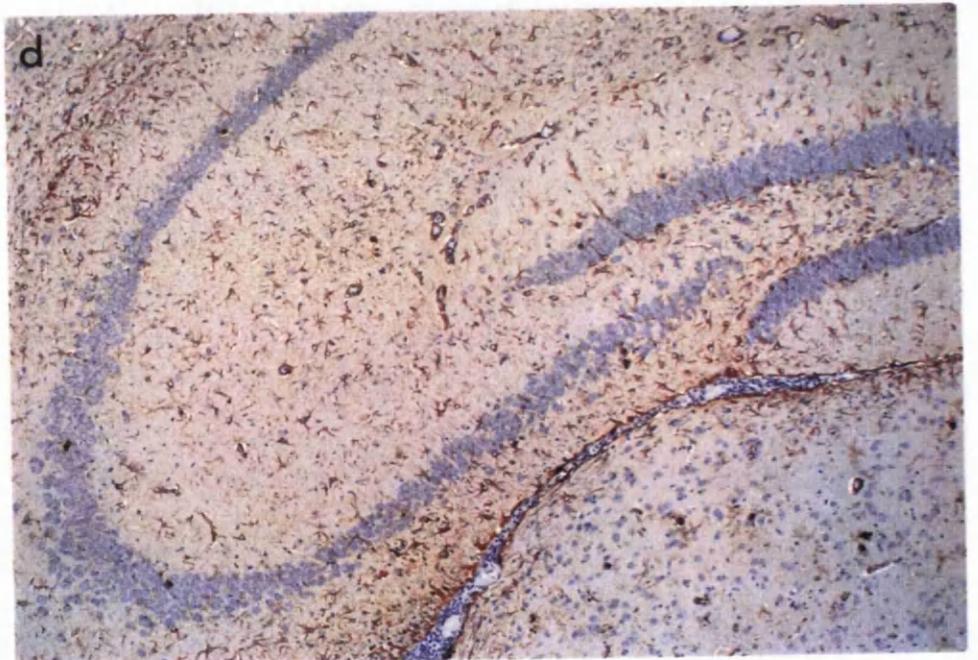
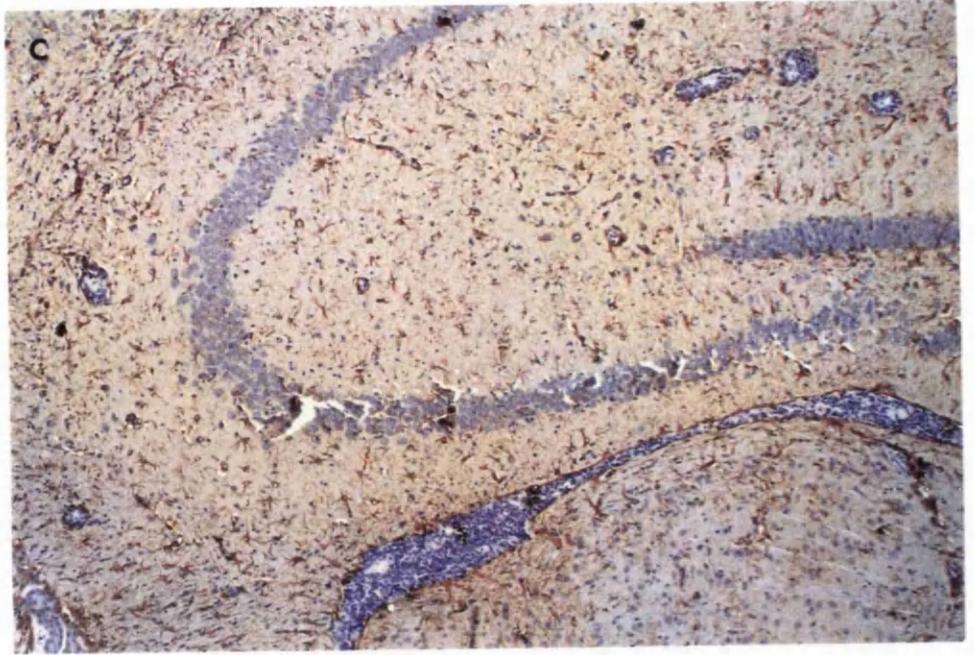
Azathioprine, at 10 $\mu$ g/ml, neither induced the expression of mRNA transcripts for any of the cytokines studied, nor did it inhibit the LPS-induced expression of these cytokine transcripts by astrocytes when it was introduced simultaneously with 10 $\mu$ g/ml LPS (Figures 6.4a, b and c).

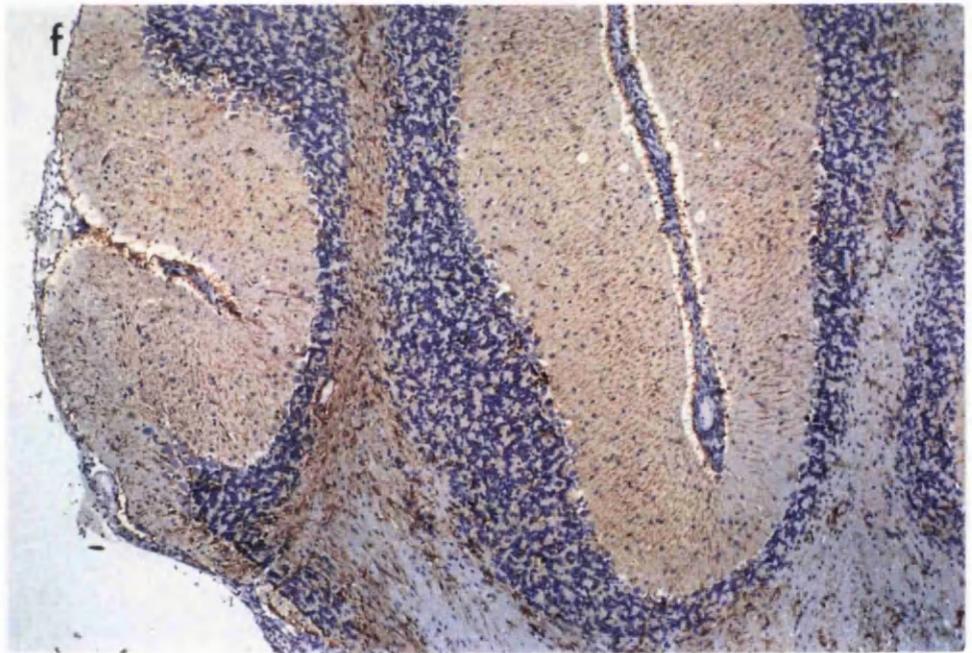
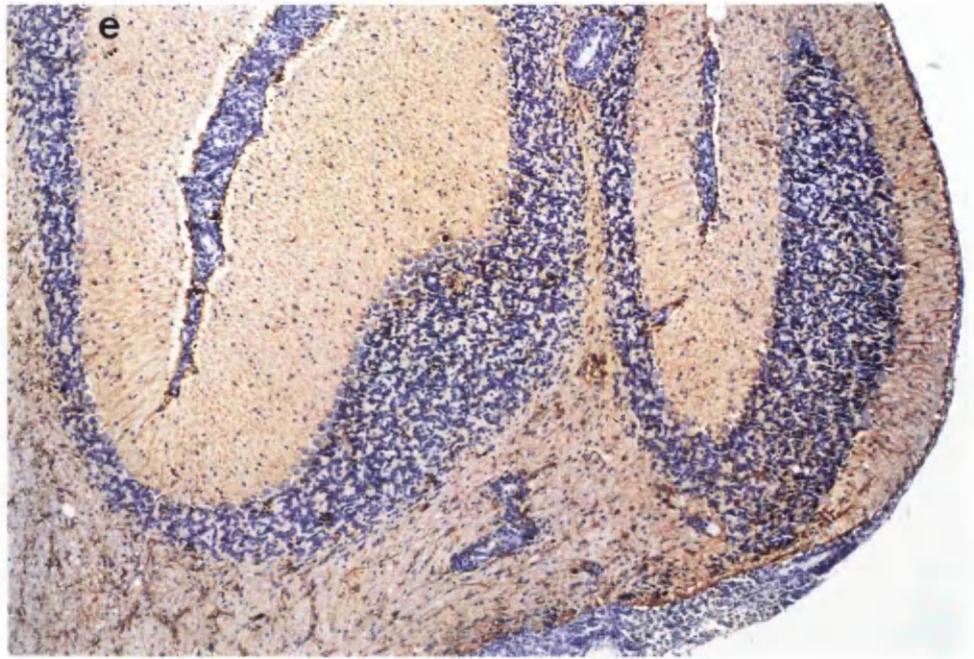
### Figure 6.3

The effect of azathioprine on the CNS pathology in mice treated with Berenil on day 21 after infection with the eflornithine-sensitive *T.b.brucei*, and again after relapse of parasitaemia. The sections shown in Figure 6.3a, c, and e were from a mouse in group BC3, which was sacrificed 6 days after the second Berenil, while the sections shown in Figure 6.3b, d and f came from a mouse in group A3, which received azathioprine for 7 days from the day of the second Berenil injection and was sacrificed at the end of azathioprine therapy. As a result of azathioprine treatment, there is a marked reduction in the inflammatory cell infiltration, but little reduction in the intensity of GFAP stain in the cerebral grey mater (compare a and b) and no noticeable change in the state of astrocyte activation in the hippocampus (compare c and d) and the cerebellum (compare e and f). The sections were stained with rabbit anti-GFAP antibody developed with a peroxidase/DAB kit (Vectastain) and counterstained with haematoxylin.

x90



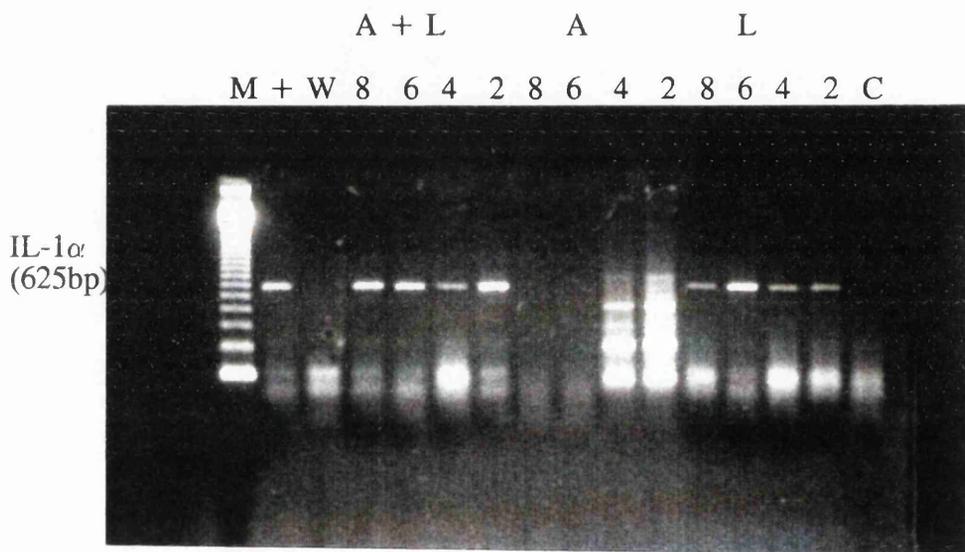




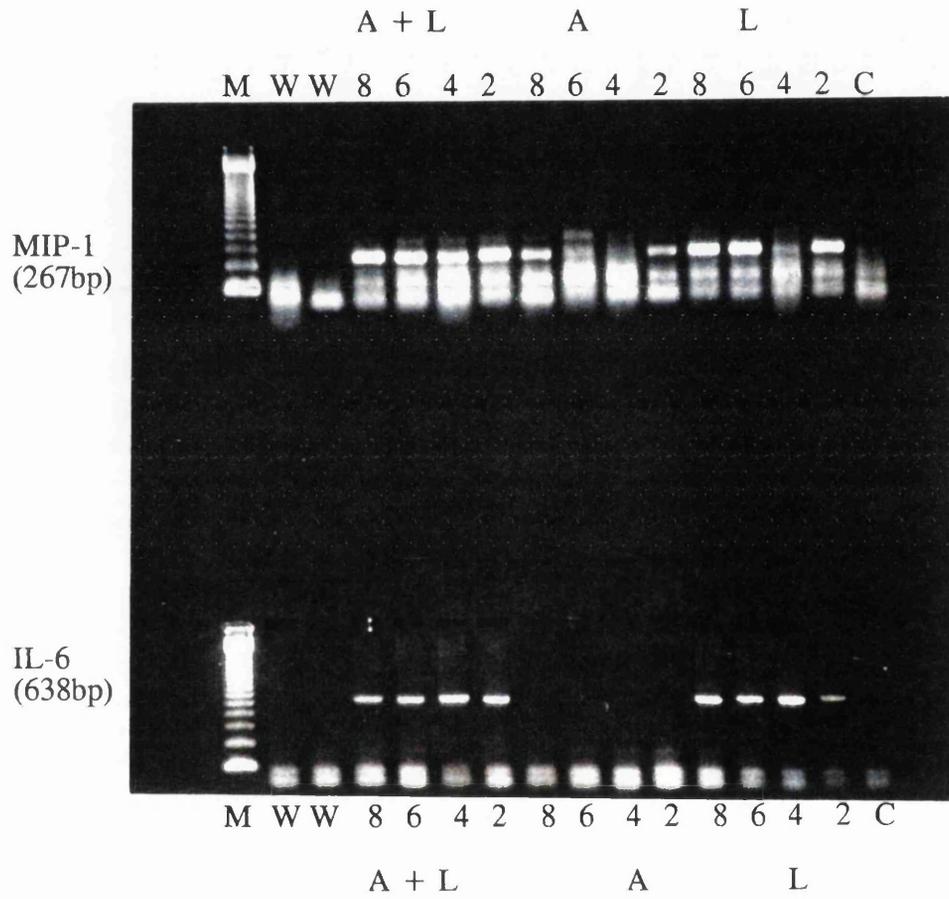
#### Figure 6.4

Gel electrophoresis showing transcripts for the cytokines: a) IL-1 $\alpha$  b) MIP-1 and IL-6 and c) IL-1 $\beta$  and GM-CSF expressed by cultured astrocytes after exposure to 10 $\mu$ g/ml LPS (L), or 10 $\mu$ g/ml azathioprine (A), or a combination of LPS and azathioprine (A+L). Azathioprine has no effect on LPS-induced expression of cytokine transcripts. The numbers 2, 4, 6 and 8 indicate time in hours after addition of stimulant, M = 123bp ladder (Gibco); + = positive control; W = water, used as a negative control; C = unexposed cells.

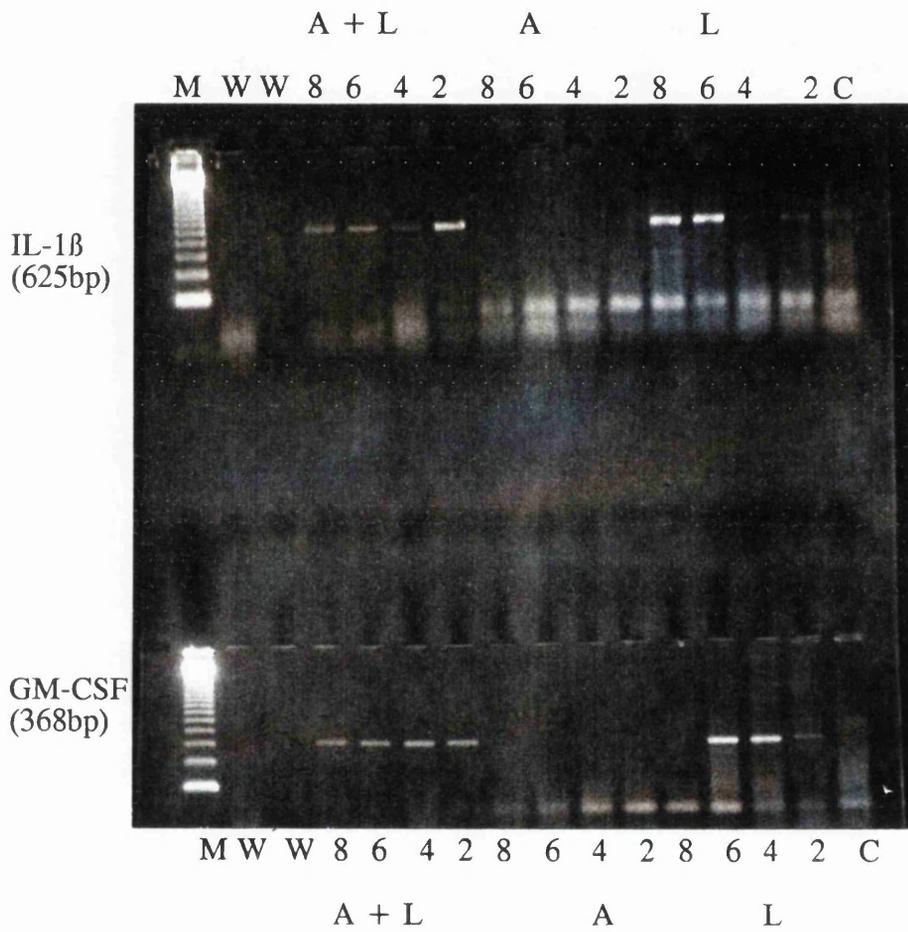
a)



b)



c)



## 6.4 DISCUSSION

The results presented here show varied effects of azathioprine on the PTRE in *T.b. brucei*-infected mice. The varied effects seemed to depend on the time at which azathioprine was administered. Thus, when azathioprine was administered prior to and at the time of PTRE induction, it prevented the development of inflammatory cell infiltration and appeared to arrest astrocyte activation when compared to the Berenil treated controls. This suggested that by inhibiting the infiltration of inflammatory cells azathioprine indirectly prevented the progress of astrocyte activation, but unlike eflornithine did not reverse the activation that had already taken place. Indeed, the results of this study showed that the astrocyte activation after 7 or 14 days of azathioprine administration, starting from the day of PTRE induction (day 26 after infection when Berenil is injected), was just slightly more than in the activation seen 32 days after infection in untreated mice, but less than the Berenil-treated controls. These results lend support to the hypothesis raised in this thesis that the infiltrating inflammatory cells produce the additional signal (probably cytokines), to provide the threshold for diffuse astrocyte activation.

The results of this study further showed that azathioprine administration at the time of the second Berenil injection removed the infiltrating inflammatory cells but did not appear to affect the astrocyte activation. Moreover, the *in vitro* studies presented here, demonstrated that azathioprine at a concentration known to inhibit cultured lymphocyte functions, had no effect on the ability of LPS-induced astrocytes to produce cytokines. Together, these results suggested that azathioprine had no direct inhibitory effect on astrocytes. Other workers (Rosenthale *et al*, 1969; Babington and Wedeking, 1971), using an experimental allergic encephalitis- (EAE)-rat model, reported that azathioprine prevented the clinical development of the disease but that the microscopical neural lesions were not prevented. Since the neural lesions in EAE consist of demyelination and gliotic scarring, these microscopic findings

support the finding in this study that azathioprine does not directly inhibit astrocyte activation.

Previous workers have reported that azathioprine prevents and reverses the mononuclear inflammatory cell infiltration into the CNS of *T.b.brucei*-infected mice (Jennings *et al*, 1989; Hunter *et al*, 1992b). However, the results of this study using the intensity of staining of the astrocyte specific marker, GFAP, showed that azathioprine neither prevents nor reverses astrocyte activation. The current study further demonstrated that azathioprine had no effect on the *in vitro* production of cytokines by LPS-stimulated astrocytes. Taken together these findings indicated that azathioprine had only a partial effect on prevention and amelioration of PTRE. This is not surprising since this drug is a specific inhibitor of lymphocyte functions. Evidence for a special effect of azathioprine on the lymphocyte population includes: 1. An inhibitory effect on the primary antibody response to T cell-dependent antigens, such as, the heterologous red-blood cells (Nathan, Beiber, Elion and Hitchings, 1961), tetanus toxoid (Friedman, Gelfand and Bernheimer, 1971) candida (Kakuk, Balogh, Szabolcsi and Halmy, 1972), human serum albumin (Meyer zum Buschenfelde and Freudenberg, 1969) but not to T cell-independent antigens, such as the pneumococcal polysaccharide (SIII). 2. The inhibition of *in vitro* T-lymphocyte rosette formation around heterologous RBCs (Vas and Lowenstein, 1965; Bach and Bach, 1972), the cytotoxic capacity of *in vivo*-stimulated rat lymphocytes (Wilson, 1965), production of specific antibody by lymphoid cells following *in vitro* but not *in vivo* sensitisation (Rollinghoff, Schrader and Wagner, 1973). 3. The inhibitory *in vivo* effects on the lymphocyte behaviour, its synergisms with thymectomy (House, Boak and Draper, 1974) and the inhibition of the effector capacity of T cells in azathioprine-treated patients (Dawkins and Mastaglia, 1973; Richens, Williams, Gough and Ancill, 1974a; 1974b).

When azathioprine and its active metabolite, 6-mercaptopurine (6-MP), are appropriately administered during the primary immune response, normal development of immunological memory (as judged by normal booster reaction) may be impaired even to the extent of producing temporary but specific immune paralysis or tolerance (Nathan *et al*, 1961; Schwartz, 1965), with azathioprine showing greater potential for immunosuppression than 6-MP (Nathan *et al*, 1961). The results presented here showed an absence of inflammatory cell infiltration into the CNS when azathioprine was administered just before or at the time of PTRE induction. Since the infiltrating cells in PTRE of *T.b. brucei*-infected mice consist mainly of lymphocytes and plasma cells, it is possible that azathioprine acted on these cells causing temporary immune paralysis, thus preventing the infiltration and proliferation of lymphocytes in the CNS at this time.

The timing of azathioprine administration seems to be critical since it appears to have an effect on the induction phase of immune response but not on an already established response. The results of this study confirm this, as it was found that administration of azathioprine at the time of PTRE induction prevented infiltration of inflammatory cells while it was ineffective when administered 7 days after induction of PTRE. Similar observations have been made in various experimental procedures: azathioprine stops the enlargement of germinal centres and lymphoid follicles in lymph nodes and spleen of rodents during the first set reaction but not the second set reaction of graft rejection (Marmor, 1967; Skopinska, Rychlikowa and Nouza, 1968; Laden, 1972; Swingle, Grant and Valle, 1973); and it (azathioprine) is highly effective in preventing the development of clinical EAE in rats when given during the first 7 days and less so when given later (Babington and Wedeking, 1971). Moreover, the presence of azathioprine within the first 24 hours of lymphocyte culture was found to be necessary for the *in vitro* inhibition of: a two-way mixed leukocyte reaction (Vas and Lowenstein, 1965; Bach and Bach, 1972), production of

specific antibodies after *in vitro* stimulation of lymphocytes (Rollinghoff *et al.*, 1973) and cytotoxic capacity of cultured rat lymphocytes after *in vivo* stimulation (Wilson, 1965). Azathioprine and 6-MP can also abolish or delay the primary humoral response to both heterologous cellular, or soluble protein antigens, inhibiting the switch from IgM to IgG production; the prolongation of IgM production being attributable to a lack of normal feedback control (Uhr and Braumann, 1961). In multiple sclerosis patients, azathioprine has been reported to slow down the clinical deterioration associated with this disease, the effect being more marked in patients who had the disease for less than 2 years. Patients who had been affected for longer durations did not show any significant reduction in the rate of disease progression (Patzold and Pocklington, 1980; Muller, Saueremann and Kreiner, 1981)

This study showed that administration of azathioprine starting 7 days after Berenil treatment, neither reversed the cellular infiltration nor the astrocyte activation in the resultant PTRE in *T.b. brucei*-infected mice. It is possible that at this time, the lymphocytes had proceeded into the secondary phase of immune response and were insensitive to azathioprine. Furthermore, macrophages which occur secondary to lymphocytes in an immune response and which might not respond to azathioprine (Gotjamanos, 1971; Kaufman and McIntosh, 1971), could have infiltrated the inflammatory foci by this time.

The observation, in this study, that azathioprine almost entirely stopped the infiltration and proliferation of lymphocytes when given at the time of the second Berenil treatment, seemed to suggest that the lymphocytes were still in the induction-phase response even at this time. This was not surprising considering that trypanosomes undergo antigenic variation, which could ensure that lymphocytes are constantly in the induction phase, reacting to the new antigenic type of trypanosomes. The observation that delaying azathioprine administration for 7 days after Berenil removed this effect on lymphocytes, and

knowing that Berenil clears parasites from the circulatory system, suggested that the new antigenic types emanate from the systemic circulation to constantly present a new challenge to the infiltrating lymphocytes. It would appear that when the systemic circulation was cleared of parasites by Berenil, and lymphocytes given time to accommodate to the trypanosomes in the CNS, they proceeded to the secondary phase of response becoming insensitive to azathioprine. An alternative possibility is that the immunosuppression that occurs during trypanosome infections affects the lymphocytes in such a way that they are arrested in the induction phase.

Administration of azathioprine at the time of the second Berenil injection cleared the bulk of the infiltrating cells but a few inflammatory foci remained. Most of the remaining cells were macrophages which are a component of the infiltrating cells in the trypanosome-induced CNS lesion and PTRE, and which might not respond to azathioprine. The effect of azathioprine on macrophages is controversial. Some authors found azathioprine to have no effect on the ability of activated macrophages to phagocytose particulate matter such as carbon (Gotjamanos, 1971) or colloidal gold (Kaufman and McIntosh, 1971). Other workers, have shown that 6-MP has a suppressive effect on the ability of macrophages to phagocytose  $^{14}\text{C}$  and their ability to migrate from capillary tubes regardless of changing macrophage inflammatory factor (MIF) concentration (Philip and Zweiman, 1973).

When the course of azathioprine therapy was extended to 14 days, there was a marked reduction in the infiltration of inflammatory cells and a slight reduction in the astrocyte activation. It is possible that this reduction in astrocyte activation was due to the reduced stimulatory boost from the disappearing inflammatory cells, since the *in vitro* studies confirmed that azathioprine had no direct effect on astrocytes. It would appear that prolonging the course of azathioprine therapy might be useful in the amelioration of an established PTRE. Unfortunately, at the doses used in this study (100mg/kg,

lower doses were ineffective), the drug was poorly tolerated, with most of the mice dying before the completion of the 14-day course. Similar intolerance of azathioprine has been reported in Rhesus monkeys in the prevention of renal allograft-rejection (Dicke *et al*, 1971) and in the treatment of arthritis in rats (Jouanneau, Brouilhet, Kahan, Charles and Delbarre, 1973). Long term (1-5 years) azathioprine therapy has been reported in multiple sclerosis patients (Patzold and Pocklington, 1980; Muller *et al*, 1981; Milanese, La Mantia, Salmaggi, Campi, Bortolami, Tajoli, Nespolo and Corridori, 1988). The outcome of these studies were conflicting: while Milanese *et al* (1988) reported a significant reduction of disease progression in azathioprine treated patients (favourable outcome seen only after 3 years of therapy), other researchers found a high incidence of clinical deterioration, with 64.1% of these patients developing severe side effects (Muller *et al*, 1981). The side effects included gastric complaints, considerable leukocyte depression, oedema, alopecia, strumata, increase in transaminases, recrudescence of mycosis and pyoderma (Muller *et al*, 1981).

The observation here that azathioprine did not reverse the astrocyte activation, and that cellular infiltrates recurred within 7 days of discontinuation of azathioprine therapy (compared with 14 or more days after eflornithine, a drug that stops astrocyte activation) seems to support the hypothesis proposed earlier in this thesis that activated astrocytes initiate inflammatory cell-infiltration into the CNS. These results further suggest that the effects of azathioprine on lymphocytes are short lived, with the result that lymphocytes become active and proliferate as soon as azathioprine therapy is withdrawn. Indeed it has been reported that a burst of mitotic activity occurs shortly after therapy with azathioprine is stopped (Skopinska *et al*, 1968).

It would appear that administration of azathioprine for the prevention of PTRE would require an exact knowledge of the stage of the inflammatory

lesion in the brain. Using the current tools of diagnosis, it is impossible to know the exact stage of the reaction in the CNS of sleeping sickness patients and any improvement would depend on a chance "correct" timing. However, the use of azathioprine at the time of trypanocidal therapy could be beneficial since administration at the time of Berenil treatment (even after relapse of parasitaemia) seemed to slow down the progress of PTRE. It is possible that azathioprine therapy might arrest the development of clinical PTRE, especially when used in combination with the first-time trypanocidal treatment (i.e. non-relapsed patients).

In experimental *T.b. brucei* infections of mice, it has been observed that the use of three consecutive doses of azathioprine at the time of the first Berenil treatment reduced the incidence of hind-limb paralysis to 25% from 50% in Berenil treated controls (F.W. Jennings, personal communication). This seems to suggest that the beneficial effects of azathioprine could be utilised without prolonging the duration of therapy and avoiding the accompanying adverse side effects. While azathioprine might not reverse the already present pathological changes during a relapse infection, its simultaneous use with a trypanocidal agent might prevent development of clinical PTRE.

It has been reported that in the prevention of graft rejection, azathioprine works best at safe dosages when used in triple-combination with other anti-inflammatory drugs especially corticosteroids and cyclosporin A (Vereerstraeten, 1993; Isoniemi *et al*, 1993). This possibility has not been investigated in models of trypanosomiasis. One wonders whether investigating this possibility would be worthwhile considering that the combined anti-inflammatory therapy, would have to be used in conjunction with trypanocidal drugs, in subjects that are likely to be already immunosuppressed.

## **CHAPTER 7**

### **GENERAL DISCUSSION AND CONCLUSIONS**

This thesis concerns the role of astrocytes in the pathogenesis of CNS trypanosomiasis and in the encephalopathy that results from treatment of late-stage trypanosomiasis with drugs such as melarsoprol in HAT and Berenil in experimental *T.b. brucei* infections in mice. The working hypothesis of the present investigations, was that astrocytes initiate and propagate the CNS pathology during trypanosome infections and in PTRE. The objectives of the study were: to determine when astrocytes become activated during the course of trypanosome infection in mice and during curative and subcurative trypanocidal therapy; how astrocytes respond to trypanosomes *in vitro* with respect to cytokine production; and how drugs, reported to attenuate the inflammatory cell infiltrations during PTRE, affect astrocyte activation *in vivo* and *in vitro*.

Astrocyte activation, occurred initially where the blood-brain barrier is incomplete, at sites where early trypanosome invasion has been reported to occur, and was followed by infiltration of inflammatory cells into the choroid fissure, perivascular spaces and the meninges. This suggests that astrocyte activation is the first CNS response to invasion by trypanosomes and that the activated astrocytes initiate the inflammatory cell infiltration, possibly by producing inflammatory mediators. The correlation of the early astrocyte activation, with cytokine production, the knowledge that astrocytes are capable of producing cytokines and that cytokines initiate meningeal inflammation, would suggest that cytokines, produced by the activated astrocytes, initiate leukocyte extravasation into the brains of *T.b. brucei*-infected mice.

Histological examination of the brains of mice with PTRE, caused by subcurative treatment with Berenil, revealed pathology similar to that in infected untreated mice, with the exception that it occurred faster and was more pronounced. A second Berenil treatment made the lesions even more pronounced and did not kill the trypanosomes in the brain. This suggested that the cells and mediators of inflammation in infected untreated and in

subcuratively treated mice were the same. It was proposed that PTRE is an enhanced reaction to persistent infection within the brain, resulting from a temporary disruption of the blood-brain barrier, and subsequent influx of metabolites and inflammatory mediators from the circulatory system. The blood-derived metabolites and inflammatory mediators facilitate trypanosome survival, and enhance inflammation and astrocyte activation within the CNS.

Pure cultures of astrocytes responded to trypanosomes, trypanosome lysate and VSG by expressing mRNA transcripts for the cytokines, IL-1 $\alpha$  and - $\beta$ , IL-6, TNF $\alpha$ , MIP-1 and GM-CSF, indicating that astrocytes are likely to be a major source of these cytokines in the brains of *T.b. brucei* infected mice; and that trypanosomes within the brains of such mice are a potent stimulus for astrocyte activation. Production of IL-1 and TNF in the CNS would act on endothelial cells, microglia and astrocytes themselves, causing: migration, proliferation, induction of adhesion molecule expression, and production of leukocyte chemotactic cytokines. The overall effect of such cytokines would lead to extravasation of leukocytes into the CNS, proliferation of the infiltrating inflammatory cells, microglia and astrocytes, and production of more cytokines, essentially creating a self-perpetuating lesion.

Administration of eflornithine prior to and around day 21 of infection with an eflornithine-resistant trypanosome stabilate, prevented the occurrence of astrocyte activation and the subsequent inflammatory cell infiltration. When eflornithine was administered during an existing PTRE, induced by a single or double Berenil treatment, there was regression of both inflammatory cell infiltrations and astrocyte activation. These results suggested that eflornithine had an inhibitory effect on both the inflammatory cells and the astrocytes. The finding that eflornithine, inhibited the LPS-induced expression of cytokine transcripts by cultured astrocytes confirmed a direct effect of eflornithine on astrocytes. This suggested that the disappearance of astrocyte activation in the brains of eflornithine-treated, *T.b. brucei*-infected mice was not a consequence of

the disappearing inflammatory cells. Since the trypanosomes used in this study were eflornithine resistant, the remarkable reversal of the CNS pathology in the face of persistent infection demonstrates the extraordinary potential of eflornithine therapy in the late stages of African trypanosomiasis. Therefore, eflornithine may be used alone, for the treatment of trypanosomiasis, when the infecting trypanosomes are sensitive, and in combination with other trypanocides when the infecting trypanosomes are refractory to eflornithine. In addition, eflornithine would be of use in the prevention and, possibly, reversal of PTRE.

Treatment of mice with azathioprine, after day 21 of infection with *T.b. brucei* or just prior to treatment of such mice with Berenil, prevented the infiltration of inflammatory cells but did not prevent the onset of astrocyte activation. Administration of azathioprine during an established PTRE (starting 7 days after Berenil treatment) did not affect the astrocyte activation and only slightly reduced the number of infiltrating inflammatory cells. It was further shown that azathioprine did not affect the LPS-induced expression of cytokine transcripts *in vitro*. Azathioprine is a known inhibitor of lymphocytes, and, as the results in chapter 6 show, does not affect astrocyte activation either at the onset or after it has occurred. This would suggest that the onset and mediation of astrocyte activation during *T.b. brucei* infections in mice is not dependent on inflammatory cells, supporting the hypothesis that astrocyte activation is the CNS response to trypanosomes. The effect of azathioprine on inflammatory cells, depended on the time it was administered: when administered just prior to the first Berenil, or at the same time as the second Berenil, it prevented the infiltration of inflammatory cells. In such situations, there was a slight decrease in the GFAP staining intensity, reflecting a slight reduction in astrocyte activation. It was suggested that this reduction in astrocyte activation, resulted from the loss of stimulatory boost from the disappearing inflammatory cells and

not from a direct effect of azathioprine on astrocytes. It would appear that administration of azathioprine at the time of trypanocidal therapy might prevent development of clinical PTRE and by inference the development of RAE in man.

In conclusion, these studies show: 1. that the inflammatory cell infiltration during trypanosome infections in mice is a secondary event to a response from within the CNS, 2. that astrocytes play an important role in this initial CNS response to trypanosomes and in inducing the subsequent leukocyte infiltration and proliferation, through production of inflammatory mediators, including cytokines, and 3. that eflornithine, a drug that affects both the astrocytes and the infiltrating inflammatory cells is more potent in preventing the Berenil-induced PTRE in mice than azathioprine, a drug that affects only the inflammatory cells. It would appear that identifying the mediators (such as cytokines) of CNS inflammation; and specifically inhibiting these mediators would give a better prognosis than using anti-inflammatory agents that affect a specific cell type. Future studies using specific cytokine inhibitors, such as anti-cytokine antibodies or cytokine-receptor antagonists, would be very useful in addressing this question. Eflornithine might also be useful in other inflammatory conditions such as bacterial meningitis and viral meningitis. Indeed, the *in vitro* results presented in this study showed that eflornithine reverses the LPS-induction of cytokine production by astrocytes. Although the cell infiltrations in bacterial meningitis consist mainly of polymorphonuclear cells, the cytokines produced by astrocytes, some of which were discussed here, would also have an activating and proliferative effect on those cells.

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