THE REGULATION OF PLEOMORPHIC *TRYPANOSOMA BRUCEI* INFECTIONS IN IMMUNOCOMPETENT HOSTS

Laura Margaret Louise McLintock B.Sc.

Department of Zoology

University of Glasgow

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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DECLARATION

The results presented in this thesis are my own work except where there is an explicit statement to the contrary.

The work described in Chapter 3 has been accepted for publication: McLintock, L.M.L., Turner, C.M.R. & Vickerman, K. A comparison of multiplication rates in primary and challenge infections of *Trypanosoma brucei rhodesiense* bloodstream forms. *Parasitology* (IN PRESS). Some of this work has already been published as a meeting abstract: McLintock, L., Turner, C.M.R. & Vickerman, K. (1988). Evidence that host factors do not control replication rates in pleomorphic *Trypanosoma brucei rhodesiense* infections. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 82, 940 (abstract).

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SUMMARY

This thesis describes work conducted to elucidate mechanisms by which mammals control pleomorphism in bloodstream populations of *Trypanosoma brucei*.

The hypothesis that division of *T. brucei* slender bloodstream forms is dependent upon the availability of a host-derived growth factor was tested by superimposing challenge doses of slender form trypanosomes onto preexisting infections at a time during the primary infection when stumpy forms predominated. The challenge populations grew in the doubly-infected mice indicating that depletion of a putative growth factor by the expanding population of the primary infection had not limited the trypanosomes' capacity for division. This effect was independent of the variable antigen type (VAT) of the trypanosomes and of their stock of origin.

In assays of antibody-dependent complement-mediated lysis *in vitro* slender form parasites were lysed between 2.5 and 15 times more rapidly than stumpy forms. In assays of attachment of opsonised parasites to peritoneal macrophages *in vitro* the majority of both slender and stumpy form parasites attached to the macrophages in the presence of specific antibody, and this attachment occurred at the same rate for both morphological forms. When heat inactivated specific antiserum was used attachment did occur, indicating that Fc receptors on the macrophages mediated the attachment.

VAT-specific antibody cannot be detected in the serum of rodents infected with monomorphic parasites. This has led to the supposition that slender form bloodstream trypanosome populations do not elicit antibody responses. Experiments were conducted to determine whether

this effect is due to trypanosomes in the bloodstream of a mammalian host acting as a "sink" for specific antibody. High titres of specific antibody could be detected in serum within a few hours of drug-induced death of parasites. This antibody was newly-secreted from plasma cells that had been stimulated several days earlier; it became detectable as a free serum titre because the parasites which had been binding all secreted antibody had been removed from the circulation after drug-treatment. In non-cured mice serum antibody titres became detectable later in the infection and/or were present at lower titres. Monomorphic trypanosomes kill their hosts approximately 4 days after infection, before antibody titres are sufficiently high for free serum antibody to be detected. Therefore serum antibody titres are not reliable indicators of an anti-trypanosome immune response *in vivo*.

LIST OF ABBREVIATIONS

AnTat	Antwerp *Trypanozoon* antigen type
BIIT	blood inhibition of infectivity test
cAMP	cyclic 3',5'-adenosine monophosphate
CNS	central nervous system
dd H_2O	distilled, deionised water
DEAE-cellulose	diethylaminoethyl-conjugated cellulose beads
DFMO	DL-α-difluoromethylornithine
EATRO	East African Trypanosomiasis Research Organization
EDTA	ethylene diamine tetracetic acid
EGF	epidermal growth factor
Fc	crystallisable fragment obtained after papain hydrolysis of immunoglobulin
FITC	fluorescein isothiocyanate
GUPM	Glasgow University Protozoology Monoclonal (antibody)
GUTat	Glasgow University *Trypanozoon* antigen type
HDL	high density lipoprotein
IEF	isoelectric focusing
IFT	immunofluorescent antibody test
ILTat	International Laboratory for Research into Animal Diseases *Trypanozoon* antigen type
LDL	low density lipoprotein
MEM	minimum essential medium
PBS	0.017M phosphate buffered saline, pH 7.2
PDGF	platelet derived growth factor
pI	isoelectric point
PS	phosphate buffered saline and dd H_2O in ratio 6:4
PSG	PS plus 10 g/litre D-glucose
RBC	red blood corpuscle
SE	standard error
SHAM	salicylhydroxamic acid
SIP	stock iso-osmotic Percoll
TCA cycle	tricarboxylic acid cycle
VAT	variable antigen type
VSG	variant specific glycoprotein

Chapter 1

GENERAL INTRODUCTION

1.1 THE BRUCEI GROUP OF TRYPANOSOMES

Two subspecies of *T. brucei*, *T. b. brucei* and *T. b. rhodesiense*, have been used in the experiments described in this thesis. The name *T. b. gambiense* will also be encountered in discussion of trypanosomiasis in general. These three members of the brucei group of trypanosomes were originally thought to be separate species (reviewed in Hoare, 1972). This conclusion was based on criteria such as the distribution of the trypanosomes in Africa, the nature of the disease they cause, their mammalian hosts, and the vector species. Later, the three were considered to be subspecies (Hoare, 1972), and this is still generally thought to be the case, as the names I have used indicate. The three parasites of the brucei group are now classified together as the subgenus *Trypanozoon*.

Several general observations have been made about differences between these three subspecies (reviewed in Jordan, 1985). *T. b. brucei* infects domestic and wild animals, while *T. b. rhodesiense* also infects humans; *T. b. gambiense* infects humans and pigs. *T. b. rhodesiense* causes acute disease in man, while *T. b. gambiense* causes chronic disease. *T. b. brucei* and *T. b. rhodesiense* are virulent in laboratory rodents, while *T. b. gambiense* is not. *T. b. rhodesiense* is found in East Africa, *T. b. gambiense* in West and Central Africa, and *T. b. brucei* throughout the tsetse belt of Africa. *T. b. gambiense* is transmitted by tsetse flies (genus *Glossina*) of the palpalis group of species which inhabit vegetation near rivers and streams and around settlements, while *T. b. rhodesiense* is transmitted by morsitans group flies that live in the savanna woodlands. Exceptions to all of the

statements above create difficulties in diagnosis and treatment of both animal and human trypanosomiasis, as well as in the planning of strategies to control the diseases (Jordan, 1985).

Within the brucei group *T. b. brucei and T. b. rhodesiense* are morphologically indistinguishable from each other, and in practise they are differentiated by their ability to survive the blood incubation infectivity test (BIIT) and to infect humans (*T. b. brucei* cannot survive in human serum and so cannot infect humans), and it was on this basis that they were considered to be separate subspecies (Rickman & Robson, 1970a, b). That this is an unreliable criterion for taxonomic purposes has been demonstrated by Van Meirvenne, Magnus & Janssens (1976): trypanosome populations derived from a clone could be either resistant or sensitive in the BIIT. More recently biochemical and molecular techniques have been employed in studies of these parasites. Analyses of the degree of similarity between polymorphic isoenzymes in groups of field isolates of the brucei group parasites have shown *T. b rhodesiense* and *T. b brucei* to be very similar to each other (Tait *et al.*, 1985) but significantly different from *T. b. gambiense* (Gibson, Marshall & Godfrey, 1980; Tait, Babiker & Le Ray, 1984).

This similarity of *T. b. rhodesiense* and *T. b. brucei* has been confirmed in a recent investigation of DNA polymorphism within ribosomal RNA genes (Hide *et al.*, 1990). Hide *et al.* (1990), together with two other studies using antigen gene probes (Paindavoine *et al.*, 1986, 1989), also showed that *T. b. gambiense* is different from *T. b. brucei* and *T. b. rhodesiense*. In conclusion, it is now thought that *T. b. brucei* and *T. b. rhodesiense* are host range variants of the same subspecies, whereas *T. b. gambiense* is more distantly related. Formal changes to the nomenclature, however, are still awaited.

1.2 SLEEPING SICKNESS AND ANIMAL TRYPANOSOMIASIS

1.2.1 Human sleeping sickness

Sleeping sickness caused by either *T. b. gambiense* or *T. b. rhodesiense* begins as an oedematous, trypanosome-containing swelling (the chancre) that develops in the skin at the site of a bite from an infected tsetse fly. From this site the trypanosomes disseminate to the bloodstream and initiate a systemic infection. Trypanosomes also leave the bloodstream and multiply in other body tissues and fluids including the CNS. In the acute disease, usually caused by *T. b. rhodesiense*, there is a relatively high parasitaemia, enlargement of the lymphoid tissues (particularly the spleen), haemolytic anaemia, fever, followed within a few weeks by excessive sleeping and finally coma due to the CNS involvement. In the chronic disease, usually caused by *T. b. gambiense*, the much lower levels of parasites circulating in the blood cause a prolonged period of wasting, with intermittent fever, as well as inflammatory responses in lymphoid tissues and oedema due to increased vascular permeability. In very long term infections, complications occur due to immune complex deposition, and autoimmunity may occur (reviewed in Jenkins & Facer, 1985). The neurological complications may be delayed for many months or years (reviewed in Poltera, 1985).

Epidemics of sleeping sickness due to *T. b. gambiense* have been documented within the last 100 years in the Congo basin (1896-1906) and throughout W. Africa from Senegal to Cameroon (1920-1940) (reviewed in Scott, 1970). During epidemics transmission of the disease appeared to be from human to tsetse and back to human, so that for a long time it was thought that humans were the only mammalian host of *T. b. gambiense*. It is now known that *T. b. gambiense* infects both domestic animals (pigs, dogs) (Denecke, 1941) and wild animals

(hartebeest, kobs) (Gibson *et al.*, 1978), and that these species probably maintain the parasites between human epidemics. It is not known what triggers the change from endemic low levels of sleeping sickness to epidemic spread of disease: probably many factors govern the rate of infection in the flies, and the rate of transmission between flies and mammals (Jordan, 1985).

The species of tsetse flies that transmit *T. b. gambiense* (members of the palpalis group, including the species *G. palpalis*, *G. fuscipes* and *G. tachinoides*) prefer habitats near flowing water, where thickets of vegetation occur. In rural Africa they overlap significantly with human settlements (Nash, 1948), also near streams and often built within bush thickets for protection. This means that all people in these areas are equally at risk of being bitten by an infected fly. Current active foci of sleeping sickness due to *T. b. gambiense* are in Mali, Ivory Coast, Cameroon, Central African Republic, southern Sudan, Republic of Congo and Zaire (WHO/FAO, 1979; WHO, 1987).

The disease caused by *T. b. rhodesiense* is much more severe in humans than the disease caused by *T. b. gambiense*, and can be fatal in a few weeks if untreated. It reaches epidemic scale in relatively small, discrete areas within the total area of Africa inhabited by tsetse flies (e.g. recently in Ethiopia; McConnell, Hutchinson & Baker, 1970), and a smaller total number of deaths are caused by *T. b. rhodesiense* than by *T. b. gambiense*. There are persistent foci of the disease caused by *T. b. rhodesiense* in Botswana, Zambia, Mozambique, Kenya, Malawi, western Tanzania and Rwanda. *T. b rhodesiense* is primarily a zoonotic infection of wild ungulates and cattle. Transmission is mainly through tsetse flies of the morsitans group of *Glossina* species (including the species *G. m. morsitans*, *G. m.*

centralis and *G. pallidipes*) that inhabit savanna woodland. In general, humans are at risk of infection only when they intrude (e.g. as hunters or tourists) into such areas.

In the early 20th century it was thought that the brucei-group parasites infecting humans and wild animals in E. Africa were confined strictly within their host species. A long term experiment begun in the 1930s in the Tinde Laboratory, showed that this is not the case (Corson, 1936). A tsetse fly was infected by being allowed to feed on a *T. b. rhodesiense*-infected human sleeping sickness patient. The fly was subsequently able to infect sheep and antelope. The infection was cyclically transmitted through sheep for 23 years, and in an experimental infection of a human volunteer (!) was shown still to be human-infective (Willet & Fairbairn, 1955). Previously, a human *T. b. rhodesiense* isolate had been shown to infect Zebu cattle (Wilde & French, 1945) producing an infection that lasted 6 months before self cure occurred. Similarly, Heisch (Heisch, McMahon & Manson-Bahr, 1958) inoculated himself with a brucei-group parasite isolated from a bushbuck and showed it to be human-infective. Thus although *T. b. brucei* is unable to infect humans, *T. b. rhodesiense* has a huge animal reservoir through which to circulate.

It is estimated that 45 million people in Africa are at risk of contracting sleeping sickness, but that only 10 000 new cases are reported each year (WHO/FAO, 1979; WHO, 1987). This latter figure probably grossly underestimates the total number of cases due to the lack of mechanisms for diagnosing and reporting the disease in many areas.

The distinction between the diseases caused by the two parasites is becoming more difficult to define; exceptions to the E. African-acute

and W. African-chronic guide have regularly been observed. An example of this is the chronology of the progressive overlap of the two parasites around L. Victoria (reviewed by Duggan, 1970; Ford, 1971; Wellde, 1989). Between 1901 and 1910 there was a *T. b. gambiense* epidemic (transmitted by *G. fuscipes*) in Uganda, and this spread southwards around both shores of the lake to northern Tanzania. *T. b. rhodesiense*, which had been present in southern Tanzania, spread northwards to the southern shore of the lake (Maclean, 1930), and reached the northern shore of the lake by about 1940 where it was responsible for another epidemic in Uganda (transmitted by *G. pallidipes*). Later, *T. b. rhodesiense* was isolated from *G. f. fuscipes* in Uganda, even though *G. pallidipes* was still the main vector for this parasite (Southon & Robertson, 1961). This was followed in 1964 by outbreaks of sleeping sickness in Kenya in areas where only *G. f. fuscipes* existed. Currently the *T. b. rhodesiense* outbreak in Uganda has *G. f. fuscipes* as its main vector, the numbers of *G. pallidipes* having declined (Okoth, 1982). Further evidence of difficulties in distinguishing the two diseases comes from the isolation of *T. b. rhodesiense* (defined by molecular criteria) from patients in W. Africa (Hide *et al.*, 1990).

1.2.2 Trypanosomiasis in domestic animals

Domestic cattle can be infected with various species of trypanosome (reviewed in Jordan, 1985). Clinically, *T. (Duttonella) vivax* and *T. (Nannomonas) congolense* are the most important species involved. *T. b. brucei* also infects these animals but is not thought to cause significant pathology. Mixed infections with two or more trypanosome species also occur. In indigenous breeds of domestic cattle infected with *T. vivax* or *T. congolense* an initial acute phase is followed by a prolonged period of wasting during which few parasites can be detected

in the blood, but there is severe anaemia. CNS involvement in the terminal stages causes paralysis and coma, but is in general less important in the pathogenesis of cattle trypanosomiasis than it is in the human disease. Stress to the animals (e.g. movement over long distances, water shortage) can cause a reoccurrence of acute disease. Non-African breeds of cattle are more likely than indigenous breeds to suffer severe acute disease, with high parasitaemia and death within a few weeks (Murray, 1979).

Certain indigenous breeds of African cattle have developed "trypanotolerance" by natural selection through continuous exposure to tsetse challenge. This genetically-based innate resistance (reviewed in Murray *et al.*, 1979) is supplemented by exposure to local trypanosome population (i.e. acquired immunity), but animals can succumb to disease in areas of severe tsetse challenge or when moved to new areas where the local trypanosomes population differs antigenically. The degree of trypanotolerance varies between individuals (Roelants *et al.*, 1983). The main trypanotolerant breeds are the N'dama longhorns and the W. African shorthorns (both taurine breeds). The indigenous humped cattle (Zebu) do not display trypanotolerance.

1.3 LIFECYCLES OF TSETSE-TRANSMITTED TRYPANOSOMES

The lifecycles of the African trypanosomes have been extensively documented (reviewed in Hoare, 1972; Vickerman, 1985), so only a brief description is included here. The changes that occur during the lifecycle are adaptations by the parasite to maximise survival in and transmission between the various host environments. The pathogenic African trypanosomes are cyclically transmitted between mammalian hosts and tsetse flies. A basic pattern in the parasite lifecycle is

that dividing and non-dividing stages alternate: the non-dividing stages are transmission or migrating forms, and the dividing forms establish the infection in the new environment. Several morphological types can be distinguished in both hosts, and these types also differ in their biochemical processes, particularly in regard to nutrition and protection against host defence mechanisms.

Infections in mammals are initiated by intra- or sub-dermal inoculation of metacyclic trypanosomes in the saliva of the tsetse fly as it feeds. These metacyclic forms transform into long slender bloodstream forms as they migrate from the skin to the bloodstream via the draining lymphatics. Slender forms divide rapidly and are responsible for the rapid increase in parasitaemia seen in the bloodstream. They metabolise glucose, which is freely available in mammalian bloodstream, in glycosomes (Opperdoes & Borst, 1977), organelles that are thought to be exclusive to the Order Kinetoplastida (Opperdoes, 1982). The pyruvate produced from the oxidative catabolism of glucose is excreted, rather than oxidised as in mammalian cells. This is because the mitochondrion, site of the tricarboxylic acid (TCA) cycle, is repressed in the slender forms and appears acristate in electron micrographs (Vickerman, 1965). The usual cytochrome-mediated electron transport processes are also inactive in the mitochondrion so bloodstream trypanosomes reoxidise the NADH produced in glucose catabolism using a novel enzyme complex, the glycerol-3-phosphate shunt, that is not present in mammalian cells (Bowman & Flynn, 1976; Opperdoes *et al*, 1977).

Slender forms differentiate through a continuum of intermediate forms to the short stumpy forms. These forms do not divide and are infective to the tsetse fly vector. If taken up by a tsetse fly in a blood meal they will survive and continue the lifecycle. In these forms partial

activation of the mitochondrion has occurred, and enzymes necessary for amino acid based metabolism and a cytochrome-based electron transport chain are active in the mitochondrion.

Bloodstream forms ingest soluble proteins via their flagellar pocket, probably by a receptor-mediated process (Coppens *et al.*, 1987). Stumpy forms have higher rates of endocytosis and greater lysosomal activity than slender forms (Langreth & Balber, 1975). Possibly, stumpy forms are catabolising glucose to a lesser extent than slender forms, instead using amino acid catabolism for energy production: this is not known to have been investigated directly.

Once in the tsetse midgut, stumpy form parasites rapidly transform into procyclic forms. The insect midgut after feeding is rich in semi-digested blood proteins and the trypanosomes' main nutrient sources are amino acids, particularly proline. Thus the mitochondrial preadaptation of stumpy forms ensures their survival in the midgut while transformation to procyclic forms occurs. Procyclic forms divide rapidly to establish large numbers of parasites in the new host and also migrate anteriorly to the proventriculus (part of the tsetse gut). The proventricular forms do not divide but also migrate forward to the mouthparts of the tsetse, and in the case of the *T. brucei*-group parasites into the salivary gland. Once there the parasites, now of epimastigote morphology, attach to the salivary gland epithelium by their flagella and divide. Epimastigotes then transform to non-dividing, mammal-infective metacyclic forms through a series of intermediate stages (Tetley & Vickerman, 1985).

The mature metacyclic forms, slender forms and stumpy forms are all coated with a compact layer of a single molecular species of variant surface glycoprotein (VSG) (Vickerman, 1969; Cross, 1975). This VSG

molecule is the target of specific immune attack in the mammalian hosts, and has evolved in trypanosomes as a mechanism of protecting the parasites from specific and non-specific host defence mechanisms against the plasma membrane and its associated proteins. The parasite population as a whole escapes the specific response produced against a VSG because individuals in the population may switch to expression of a different VSG to which specific responses have not yet been generated (Vickerman & Luckins, 1969).

The parasite stages in the tsetse fly do not express VSG. They have no need of protection against an efficient mammalian immune system. Some stage-specific glycoproteins are expressed on the membrane of procyclic forms, however, and of these, the predominant "procyclin" molecules are the focus of much current research (reviewed by Roditi & Pearson, 1990). There seems to be much less variability in procyclin molecules than in VSGs, and the former do not form a dense layer like the surface coat on the mammalian stages. Their limited variability may allow them to be exploited for development of a transmission blocking vaccine (Murray, Hirumi & Moloo, 1985).

1.4 CLASSIFICATION OF MORPHOLOGICAL TYPES OF BLOODSTREAM TRYPANOSOMES

The division of bloodstream trypanosomes into the categories long slender, intermediate, and short stumpy is to an extent arbitrary since the entire spectrum of forms between the two extremes (shown in Fig. 1.1) can be seen in stained blood smears and by electron microscopy. However, such a classification is extremely useful as an indicator of the state of a parasite population at a particular time and under particular conditions. The original classification was made by Sir David Bruce (Bruce, 1911), and Lady Bruce made definitive watercolour paintings of characteristic slender, intermediate and

stumpy forms (Bruce *et al.*, 1912). In later experiments Wijers found that not all trypanosomes he observed matched any of the morphological types very closely, and that the inclusion of two extra categories was useful: these were long intermediate (between slender and intermediate) and short intermediate (between intermediate and stumpy) (Wijers, 1959a, b; summarised in Table 1.1). This is the most detailed method available for classification of parasites examined by light microscopy, and it was used extensively in the study reported in this thesis. Trypanosomes in thin blood films were assigned to one of the five categories and then the long intermediate and short intermediate groups were divided between the two adjacent groups to give final proportions of the three main morphological types.

Trypanosome lines that have been transmitted mechanically (by syringe passage) from one rodent host to the next instead of cyclically through tsetse flies eventually produce bloodstream infections in which all the parasites are slender forms. No intermediate or stumpy forms are produced and the parasitaemia increases exponentially until the host dies. Such lines are called monomorphic. Trypanosomes lines in which differentiation occurs and the complete spectrum of morphological forms is present are termed pleomorphic.

1.5 HOST IMMUNE RESPONSES IN AFRICAN TRYPANOSOMIASIS

1.5.1 Trypanosome antigens

Antigens expressed in bloodstream trypanosomes can be divided into two categories, common and variable (reviewed in Barry, 1986b). The common antigens are expressed in all individuals in a population, although they are not necessarily common to all lifecycle stages, stocks or species of trypanosomes (Le Ray, 1975). The variable antigens differ between individual cells within a population and individuals can alter

their expression with time (Van Meirvenne, Janssens & Magnus, 1975; Esser & Schoenbechler, 1985). Bloodstream trypanosomes are defined on the basis of their variable antigen types (VATs). The physical basis of these VATs are glycoprotein molecules (VSGs) forming a continuous and compact surface coat completely covering the body and flagellum of bloodstream trypanosomes (Vickerman, 1969; Vickerman & Luckins, 1969). There are many possible VSGs, and although an individual trypanosome expresses only one at a time (Cross, 1975), switching to expression of a different one occurs in the process known as antigenic variation.

Antigenic variation is an inherent property of trypanosomes. They switch VAT in culture (Doyle *et al.*, 1980), and cloned lines that express only a single VAT at the first peak of infection in rodents will express a plethora of other VATs after just a few syringe passages at 3 day intervals (i.e. before an effective immune response can exert selective pressure on the VATs) (Le Ray *et al.*, 1977).

Trypanosome infections are characterised by successive peaks (Ross & Thomson, 1910), and a mixture of VATs is present within each peak (Van Meirvenne, Janssens & Magnus, 1975). The host immune system responds by producing specific antibodies to the VATs present in the peak, resulting in the clearance of these trypanosomes and remission of infection. A new parasitaemic peak arises when trypanosomes that escape the immune clearance (because they express new VATs) multiply.

The duration of an infection depends on the length of time the host can survive and on the number of VATs that the infecting trypanosome line can express. Host survival depends on the ability of the host to control parasitaemic peaks i.e. immunocompetence, and on the growth rate of the trypanosome line. If the host successfully controls each parasitaemic peak then the infection will continue until such time as

the trypanosomes express antigen types to which the host has immunological memory, and then self-cure occurs. Virulent parasite lines which grow at a high rate and against which an effective immune response does not develop will kill their hosts before the VAT repertoire is exhausted.

A trypanosome clone can express more than 100 VATs (Capbern *et al.*, 1977a), and up to 1000 VSG genes may be present in the trypanosome genome (Van der Ploeg *et al.*, 1982). Also, VATs are not expressed in a predictable sequence within an infection (Gray, 1965; Capbern *et al.*, 1977a; Van Meirvenne, Janssens & Magnus, 1975; Miller & Turner, 1981). These observations have precluded the development of a vaccine against bloodstream trypanosomes. Metacyclic forms were at one time considered to be a better potential target for vaccine development because they do not undergo antigenic variation and express only a limited range of VATs (Hajduk *et al.*, 1981; Turner *et al.*, 1988). Unfortunately, the metacyclic VAT repertoires of different trypanosome stocks are not identical, and changes have occurred in the metacyclic VAT repertoire of a single trypanosome stock over a period of 20 years (Barry, Crowe & Vickerman, 1983). A cocktail vaccine, therefore, would have to be tailored for use in a particular area at a particular time - not a viable proposition.

1.5.2 Specific humoral immunity

Specific IgM responses have been detected within a few days of experimental infection of rabbits with *T. b. gambiense* (Seed *et al.*, 1969), of cattle with *T. congolense* and *T. vivax* (Luckins, 1976) and of cattle with *T. brucei* (Musoke *et al.*, 1981). A humoral response to metacyclic VATs was detected in goats 2-3 days after an infected tsetse fly was allowed to feed (Barry, Emery & Moloo, 1980; Barry &

Emery, 1984). IgM levels remain high throughout the course of *T. brucei* infections in serum and cerebrospinal fluid of humans (Houba & Allison, 1966), and in cattle (Luckins, 1976). These high IgM levels have been attributed to polyclonal activation of B cells rather than to production of VAT-specific antibody (Houba, Brown & Allison, 1969; Hudson *et al.*, 1976). However, it is possible that the IgM produced in response to trypanosome infections binds to a variety of other antigens e.g. sheep RBC (Hudson *et al.*, 1976) because of cross-reactions of the trypanosome specific sera with the other antigens tested. Each VSG contains several different antigenic epitopes and there are many VATs, resulting in the production of polyspecific antiserum by the host. Another possibility is that IgM, being of low specific affinity, will bind non-specifically to heterologous antigens. Evidence against polyclonal activation of B cells comes from observations that absorption of antisera from sleeping sickness patients (Herbert *et al.*, 1980) and trypanosome-infected cattle (Musoke *et al.*, 1981) with a large range of VATs removed most of the apparently cross-reacting IgM from serum. It seems likely that many species of IgM are present for prolonged periods because the host is constantly being stimulated to produce primary immune responses to new VATs. Further evidence that IgM is specifically generated is that both natural remission and drug cure of infection cause a drop in serum IgM levels in African trypanosome infections, e.g. Berenil treatment of *T. brucei* infections of cattle (Luckins, 1976).

IgG responses to trypanosome infections are less pronounced than IgM responses both in cattle (Musoke *et al.*, 1981; Luckins, 1976) and in humans (Herbert *et al.*, 1980). Also, the IgG that is produced is less effective that IgM both *in vitro* (in agglutination, lysis and neutralisation of infectivity assays) and *in vivo* (protection of hosts

against infection) (Seed, 1972; Takayanagi & Enriquez, 1973; Luckins, 1976; Musoke *et al.*, 1981).

1.5.3 Cellular immunity

T lymphocytes proliferate in response to trypanosome antigens, *in vitro* (Campbell, Esser & Phillips, 1982; Diffley, 1983), and both helper and suppressor subsets are present (Jayawardena, Waksman & Eardley, 1978; Jayawardena & Waksman, 1977). Transient T cell proliferation has been observed in cattle infected with *T. congolense* (Morrison *et al.*, 1978) and *T. brucei* (Mayor-Withey *et al.*, 1978), although no direct T cell mediated cytotoxicity against trypanosomes has been observed. Some of the proliferating cells must be helper T cells, since IgG responses (which are T cell dependent) to trypanosome common antigens occur (Le Ray, 1975). Helper/inducer T cells have been detected in the CNS of rats with chronic *T. b. gambiense* infections (Anthoons *et al.*, 1989). Their association with class II MHC antigens or neuroglial cells suggested that a T cell dependent immune response was active in the CNS.

The necessity of these T cell responses in clearing trypanosome infections is uncertain, however, as genetically athymic "nude" mice (Campbell, Esser & Phillips, 1978; Clayton, Ogilvie & Askonas, 1979) and thymectomised, lethally irradiated adult mice (Clayton, Ogilvie & Askonas, 1979) infected with *T. brucei* controlled their parasitaemia and survived longer than immunocompetent control mice. It is possible that T cells proliferating in response to trypanosome antigens are responsible for the generalised immunosuppression that has been observed in infected animals (reviewed in Mansfield, 1981; Vickerman & Barry, 1982; Roelants & Pinder, 1983). Both initial stimulatory and later inhibitory effects of subpopulations of T cells have been observed in the chancre (Scott, 1987). These observations were not

confirmed in the local draining lymph node, however, where B cell responses were induced without accompanying proliferation of T cells, so this role for T cells seems to be confined to the chancre.

In summary, although there is evidence of T cell proliferation in response to African trypanosomes, the specific immune effector mechanisms active against the parasites can operate in the absence of functional T cells. This suggests that African trypanosomes belong in the T-independent (type II) category of antigens (see DeFranco, 1987). These antigens are characterised by repeating structure (i.e. repetitive epitopes) such as is found on trypanosome-bound VSG. T-independent (type II) antigens provoke responses in T cell deficient mice but not in B cell deficient animals.

1.5.4 Effector mechanisms of specific antibody in trypanosomiasis

Many antibody mediated effects have been observed to operate against African trypanosomes, particularly *in vitro*. Agglutination of trypanosomes by VAT-specific antibody occurs at very low antibody titres *in vitro* (Cunningham & Vickerman, 1962), and inhibition of some metabolic processes in the parasites has been reported (Thurston, 1958; Diggs *et al.*, 1976). The two anti-trypanosome effector mechanisms that appear to be most important (Vickerman & Barry, 1982) are described below.

Complement-mediated lysis of African trypanosomes

Specific lysis of trypanosomes occurs in the presence of sera from individuals or animals infected with the same parasite line (Lourie & O'Connor, 1936; Van Meirvenne, Janssens & Magnus, 1975). The importance of antibody-dependent lysis as a clearance mechanism *in vivo* is demonstrated by the ability of genetically nude mice that are deficient in several cell-mediated non-specific immune effector

functions to control a parasitaemia (Campbell, Esser & Phillips, 1978).

There is a lot of puzzling and contradictory information available concerning the roles of antibody and complement in such lysis. *In vitro*, antibody is required for lysis of bloodstream forms in the presence of complement (Diggs *et al.*, 1976). Similarly, EDTA which blocks the classical pathway inhibited lysis of *T. brucei* and *T. congolense*. However, possible alternative complement pathway mediated lysis has also been demonstrated *in vitro* (Flemmings & Diggs, 1978) in C4 deficient serum. Activated C4 is required to form the key enzyme in the classical complement pathway. *In vivo*, antibody plus complement mediated lysis of trypanosomes may be an important mechanism of clearance of parasites from the bloodstream (Capbern *et al.*, 1977b). Clearance of parasites was slower in C5 deficient mice (MacAskill *et al.*, 1980). C5 is a complement component on the part of the pathway that is common to both alternative and classical activation. However, this finding was directly contradicted (Jones & Hancock, 1983) by the finding that the survival time of infected rodents was not altered by deficiency of C5.

Opsonisation and phagocytosis of African trypanosomes

Ingestion of opsonised parasites seems likely to be a major clearance mechanism *in vivo*. *In vitro*, murine macrophages attach to and ingest *T. brucei* (Lumsden & Herbert, 1967). Mosser & Roberts (1982) confirmed that ingestion of bloodstream forms of *T. brucei* is antibody dependent whereas insect (midgut) forms activate the alternative complement pathway leading to ingestion. Attachment of opsonised parasites to phagocytes is probably mediated by Fc receptors on the phagocytic cells, since specific antibody is a prerequisite for phagocytosis

(Takayanagi, Nakatake & Enriquez, 1974a) and molecules from which the Fc region had been removed could not bind *T. b. gambiense* to phagocytes (Takayanagi & Nakatake, 1977). Similarly, decomplementation of the serum used did not abrogate ingestion of *T. b. gambiense* by cultured rat macrophages (Takayanagi, Nakatake & Enriquez, 1974b). The ingestion of *T. b. gambiense* by rabbit macrophages was enhanced, however, by addition of complement (Takayanagi *et al.*, 1987).

1.5.5 Non-specific immunity

The most striking non-specific immunity to trypanosomes is the trypanocidal effect of normal human serum on *T. b. brucei* (Laveran, 1902). This phenomenon is the basis of the inability of these trypanosomes to infect humans, and has been an established criterion for distinguishing between *T. b. brucei* and *T. b. rhodesiense* (Rickman & Robson, 1970a). The trypanolysis is probably mediated by high density lipoprotein (HDL), and is dependent on calcium ions: it is thought to be a consequence of removal of cholesterol from the trypanosome membranes (D'Hondt *et al.*, 1979; D'Hondt & Kondo, 1980).

In the absence of specific antibody phagocytic cells do not attach or ingest bloodstream trypanosomes *in vitro*, but the survival time of mice in the absence of antibody was enhanced by non-specific enhancers of phagocyte function BCG (Murray & Morrison, 1979) and *Corynebacterium parvum* (now known as *Propionibacterium acnes*) (Black *et al.*, 1989). The relationship between level of phagocytic activity and parasite clearance may be important in trypanotolerance: the trypanotolerant wildebeest have greater macrophage function than many susceptible species (Rurangirwa *et al.*, 1986).

Mice genetically deficient in macrophage function (C3H/HeJ) had similar levels of parasitaemia to control mice, but did not survive as

long (Jones & Hancock, 1983). It seems that the phagocytic system reduces the pathogenic effects of trypanosome infection even though clearance of parasites from the bloodstream is strictly dependent on the presence of specific antibody.

1.6 CONTROL OF DIFFERENTIATION

1.6.1 *1910-1960*: Relationship of differentiation to specific antibody responses

After it had been recognised that there are a number of morphological types of *T. brucei*-group trypanosomes in the mammalian bloodstream (Bruce *et al.*, 1910; Bruce, 1911), Robertson (1913a) attempted to explain their relationship to each other. She proposed that the stumpy forms present very early in infection "grew", through the intermediate stages into slender (dividing) trypanosomes, and that the daughters of division were stumpy. In *T. gambiense* infections in monkeys she observed that just before a drop in parasitaemia, the parasite population was composed almost entirely of stumpy forms (Robertson, 1912). Thus it seemed that the non-dividing, stumpy, forms were "hardy", able to survive adverse conditions occurring during a population crash, and then grow once more into slender forms capable of division.

All studies around this time supported the view that slender forms are responsible for the growth of the parasite population while predominance of stumpy forms heralds a remission: this had been shown in *T. brucei* infections in mice (Oehler, 1914a), and in *T. gambiense* infections in humans (Reichenow, 1921).

Early in the 20th century there were also reports of lysis occurring in bloodstream trypanosome populations at and after the parasitaemic peak, the so-called crisis of infection, at which trypanosomes

disappeared from the bloodstream (Massaglia, 1907; Levaditi & McIntosh, 1910; Mutermilch & Salamon, 1928). Trypanosomes in the relapse populations were resistant to the serum taken at the first crisis. In 22 relapse populations studied, 13 immunological types were found (Lourie & O'Connor, 1937).

Thus, there was generally accepted evidence that the action of antibodies controlled the number of trypanosomes, leading to remission. Growth of an antigenic variant to which the antibodies could not react caused relapse of infection. The previously-documented changes in morphology concomitant with remission and relapse were considered, therefore, also to be due to the effect of antibody (Wijers, 1957; Ashcroft, 1957). A fall in parasitaemia in rats infected with *T. gambiense* was associated with an increase of lymphocytes in the blood (Wijers, 1957), and it was concluded that the protective response was also responsible for production of stumpy forms. (This conclusion was not entirely consistent with the that of Robertson, 1913a).

Not all available evidence, however, supported the proposed antibody-mediated mechanism of induction of differentiation to stumpy forms. When rats were treated with cortisone, which inhibits production of antibodies, the proportion of stumpy forms in the infections was greatly increased (Ashcroft, 1957). Also, slow-acting drugs produced remission characterised by high proportions of stumpy forms (Oehler, 1914b). In addition, Ashcroft (1957) acknowledged that the inability of stumpy forms to divide also had a role in decrease of parasitaemia.

The work of Ashcroft (1957) with *T. rhodesiense* in laboratory rats was confirmed by Wijers (1959b) using *T. gambiense* in monkeys. The disease caused by the West African trypanosomes is chronic, so many more

cycles of infection were observed. By making several observations daily, the precise sequence of morphological changes occurring in the trypanosome populations was observed: the peak in the proportion of intermediate forms occurred immediately before the peak in the proportion of stumpy forms. He concluded that slender forms pass through a range of intermediate forms as they differentiate into stumpy forms, but did not dismiss the theory (Robertson, 1913a) that stumpy forms may also grow into slender forms after a crisis. Wijers (1959b) also suggested that the hypothesis that antibodies cause trypanosome differentiation should be tested by studying the relationship between antibody titre and the percentage of stumpy forms present.

1.6.2 *1960-1980:* Evidence that differentiation is not driven by antibody

This question of the relationship between the presence of specific antibody and induction of differentiation was addressed in detail by Balber (1972). He made careful observations of the changes in parasitaemia and proportions of morphological types in both normal and X-irradiated (immunosuppressed) mice infected with *T. brucei*. All the morphological forms normally seen in intact mice were also seen in the immunosuppressed mice, and they appeared in the same characteristic order. However, in contrast to the pattern observed in normal mice, there was no remission of infection in the immunosuppressed mice; their parasitaemia continued to rise until death. The total number of slender forms present also continued to rise throughout the infection in the immunosuppressed mice: this agrees with the previous finding that slender forms are associated with rising parasitaemia.

By converting the proportions of parasites of each type (the criteria

used by Wijers, 1957; Ashcroft, 1960; Wijers, 1959b) into absolute numbers, Balber (1972) reinterpreted the events occurring during remission in normal mice. The numbers of stumpy forms remained fairly constant during remission; the dramatic drop in total parasite numbers was mostly due to a sudden decrease in the number of slender forms. When the proportions of morphological types throughout a cycle of infection were converted into absolute numbers, it became apparent that although the *proportion* of stumpy forms increased to its maximum at the time of remission, the *number* of stumpy forms increased most rapidly during the rising phase of the parasitaemia, i.e. the numbers of all forms increased as the parasitaemia increased.

From these experiments, Balber concluded that antibody does not induce the differentiation of slender forms into stumpy forms. Supporting evidence was available: *T. brucei* and *T. rhodesiense* growing in chick embryos differentiated into stumpy forms even though no antibody was present (Goedbloed & Southgate, 1969; Goedbloed & Kinyanjui, 1970); and since Balber's report was published, differentiation has been observed *in vitro* in a lymphocyte-free culture system (Hirumi, Doyle & Hirumi, 1977). Balber concluded that antibody does alter the morphological composition of trypanosome populations in the peripheral blood, as shown by the continued presence of large numbers of slender forms and the lack of remission in infections in immunosuppressed hosts, but that it does not accomplish this by inducing differentiation. He proposed that slender forms may be more susceptible to the effects of antibody than stumpy forms, and that the antibody may alter the distribution of morphological types between the peripheral blood and extravascular tissue fluids. Slender forms are present and divide in both vascular and extravascular sites whereas stumpy forms are confined to blood vessels (Soltys & Woo, 1969;

Soltys, Woo & Gillick, 1969; Goodwin, 1971; Ssenyonga & Adam, 1975; Tanner *et al.*, 1980), and these sites have a lower antibody titre than the bloodstream (Goodwin & Guy, 1973). Thus, there may have been evolutionary pressure in favour of sequestration of slender forms, and such slender forms may constitute a pool from which the bloodstream can be reseeded after a crisis (see also Seed, Edwards & Sechelski, 1984).

1.6.3 *1980-1990:* Mechanisms that may regulate differentiation

Five mechanisms of induction of transformation from slender to stumpy forms can be envisaged (other than the action of antibodies that has been dismissed above). They are:

(1) that differentiation is a programmed event occurring after a particular number of cycles of division;

(2) that differentiation is a programmed event occurring with a defined probability per cycle of division;

(3) cell to cell contact inhibition of trypanosome division;

(4) depletion of a critical nutrient factor that is required for growth or that inhibits differentiation;

(5) production of a growth inhibitor or differentiation promoter.

Mechanisms 3, 4 and 5 would be density dependent, and mechanisms 4 and 5 could be caused either by the host in response to trypanosome infection, or by the parasites themselves.

In a given host, inoculation of varying numbers of trypanosomes of a particular line results in similar peak parasitaemia values regardless of the size of the initial inocula. This indicates that differentiation is not programmed to occur after a defined number of cell cycles (Seed & Sechelski, 1989). In addition, stumpy forms are produced at the greatest rate during the rising phase of the

parasitaemia (Balber, 1972).

A similar possibility is that there is a certain probability per cell cycle of an individual trypanosome beginning on the differentiation pathway instead of commencing another round of cell division. This would explain why stumpy forms are produced very early in infection and why they accumulate fastest during the rising phase of the parasitaemia. On its own, however, this mechanism of differentiation does not explain the observed change of predominance. To do this it would have to occur in tandem with mechanisms (4) or (5), or with selective clearance of slender forms from the bloodstream.

A parasite line can consistently reach different peak parasitaemia levels in different strains of mice (Seed & Sechelski, 1988). This suggests that contact inhibition between parasites cannot solely be responsible for induction of differentiation - a host-dependent variable would also have to be involved. Also, contact inhibition of growth is difficult to envisage mechanistically for highly motile cells that do not aggregate.

The possibility that mechanisms (4) or (5) may control differentiation has been extensively investigated by Black and colleagues. The rate of accumulation of stumpy forms in the blood varies between inbred strains of mice infected with a pleomorphic *T. brucei* line (Black *et al.*, 1983). The trypanosome population in a susceptible strain was composed of 50% stumpy forms by day 8 and 70% by day 10 post-infection, whereas over 70% of the trypanosomes in a more resistant mouse strain were stumpy by day 6. Black and colleagues addressed the possibility raised by Balber (1972) that the change in predominance was caused by selective immunological clearance of slender forms. ^{75}Se-labelled slender or stumpy form trypanosomes were inoculated into

mice that had been infected 5-7 days previously with homologous parasites. The proportion of labelled parasites remaining in the bloodstream at time-points after inoculation was calculated. The clearance rates of slender and stumpy forms did not differ from rates in control (previously uninfected) mice, and did not differ from each other. That is, the presence of a developing immune response to the primary infection did not increase the rate of clearance of slender or stumpy forms until day 7 when the clearance of both morphological types was equally enhanced (Black *et al.*, 1983). They concluded that selective clearance of slender forms did not occur, and that variation in immune capability explained the differences in parasitaemias observed between inbred mouse strains.

Thus resistance correlated with the rate at which stumpy forms come to predominate in the infection. This is because a rapid rate of accumulation of stumpy forms leads to a lower peak parasitaemia and remission after a shorter period. The fact that the same parasite line behaved differently in different hosts suggested to Black and colleagues that the rate of differentiation in *T. brucei* parasites is influenced by the host. (Black and colleagues in their publications use the term differentiation to denote a change in predominance of forms at the population level. For consistency in this thesis I have used the term differentiation only to denote changes occurring at the level of the individual parasite.) A parasite line that was monomorphic in rodents was found to produce a pleomorphic infection in cattle (Black, Jack & Morrison, 1983). When infection with this line in cattle was compared to infection, also in cattle, with a line that is pleomorphic in rodents the rates of increase of parasitaemia and the rates of accumulation of stumpy forms occurring in the two parasite populations were similar, and both stimulated specific

antibody responses (Black *et al.*, 1986). Parasites derived from the first or third parasitaemic wave in cattle infected with the rodent monomorphic line produced monomorphic infections in mice, i.e. the parasites behaved differently in the different host species. To determine whether the host exerted a positive or negative control on differentiation, Black, Jack & Morrison (1983) inoculated mice with bovine plasma from either infected or uninfected cattle. No induction of trypanosome stumpy forms occurred in the mice, therefore the trypanosome differentiation in cattle was not triggered by the presence of any serum molecule. Thus, it was proposed that differentiation is negatively controlled (Black *et al.*, 1985): that there is a host factor that either promotes division or inhibits differentiation (or both), and that such a factor is present in rodents and becomes depleted by growing trypanosome populations resulting in the predominance of stumpy forms. The fact that differentiation occurs in immunosuppressed mice indicates that the factor is not sensitive to the various immunosuppression regimes that have been used, and is not a product of cells of the immune system. Obviously, because of the differences in scale, the reverse experiment could not be performed; that is, testing if a factor present in mouse serum inhibits differentiation of pleomorphic lines in cattle.

Attempts to identify factors required for differentiation have produced equivocal results. Injection of numerous nutrients and cofactors (normal mouse plasma, foetal calf serum, ornithine, polyamines, glucose) into infected animals failed to prevent differentiation (Seed & Sechelski, 1989). This finding does not agree with the finding *in vitro* in cultures of bloodstream trypanosomes (Black & Vandeweerd, 1989) that serum lipoproteins are required for trypanosome multiplication (see next section).

The putative host-derived factor that either inhibits multiplication or promotes differentiation, as proposed by Black *et al.* (1985), could explain why different species and even strains of host animal infected with the same parasite line tolerate different parasitaemia levels before differentiation occurs (Murray, Morrison & Whitelaw, 1982). The presence of such a factor would also explain why the same parasite line causes monomorphic infections in rodents and pleomorphic infections in cattle: either cattle produce more of a factor than rodents, or produce a factor which is able to bind to receptors on that parasite line, whereas the rodent factor is not. If such a host-derived factor does exist, its concentration would be expected to vary during the course of a trypanosome infection, i.e. the concentration of a growth promoting molecule would decrease during a parasitaemic wave while that of a differentiation inducing molecule would increase. This makes it difficult to explain the maximal rate of accumulation of stumpy forms during the rising phase. It is also possible that a growth-inhibiting or differentiation-inducing factor might also be produced by the parasites themselves. The effects of any a factor should be demonstrable by study of growth and differentiation *in vitro*.

1.6.4 Role of nutrient limitation and growth factors in differentiation

In vitro, bloodstream trypanosomes required transferrin, albumin and soybean lipid in order to survive in cell and serum free medium (Black *et al.*, 1985). Under these conditions no division occurred and the parasites proceeded through the differentiation stages normally seen in the bloodstream, and if transferred to the appropriate medium once stumpy forms had appeared, could be transformed into procyclic forms. When the medium was supplemented with serum and fibroblasts (or

epithelial cells or adipocytes) division of slender forms occurred. Some of the sera tested could support division in the absence of fibroblasts, but this effect was not consistent even between different batches of serum from the same source. Fibroblasts in the absence of serum did not sustain division of slender forms.

Either high or low density lipoproteins (HDL, LDL) were required for growth of *T. brucei* to occur in culture. HDL or LDL alone could not support multiplication in the absence of some other factor(s) present in delipidated serum (Black & Vandeweerd, 1989). Trypanosomes accumulated lipids from the lipoproteins without binding, accumulating or degrading apolipoproteins. The uptake occurred at 37°C but not at 0°C, and did not required divalent ions; this indicates that uptake was probably a diffusion process rather than a receptor-mediated active transport process. Other lipoprotein associated lipids were taken up by receptor-mediated processes by slender form (actively dividing) trypanosomes, but no uptake of intact LDL (i.e. lipoprotein and lipid) was observed (Vandeweerd & Black, 1989). This latter result is in direct disagreement however, with other observations (Coppens *et al.*, 1987). Slender form trypanosomes lack several enzymes involved in *de novo* lipid synthesis because of the mitochondrial repression in these forms, and fulfil their lipid requirements by scavenging lipids from the host's bloodstream (Coppens *et al.*, 1987). Upon differentiation, activation of the mitochondrion correlates with the appearance of enzymes of lipid anabolism. These differences may explain the need of trypanosomes for lipid sources during multiplication (the exponential growth phase) and it is possible that depletion of lipid in the bloodstream of the mammalian host may trigger differentiation.

Antibodies to mammalian epidermal growth factor (EGF) receptor bind two polypeptides in extracts of *T. brucei* one of which is a 135 kDa surface molecule (Hide *et al.*, 1989) which appeared to have protein kinase activity (as does the mammalian EGF receptor). In addition, EGF was found to increase protein kinase activity of four other polypeptides in membrane-enriched fractions derived from trypanosomes. EGF increased the growth rate of*T. brucei* procyclic forms *in vitro*. The response of bloodstream trypanosomes to EGF *in vitro* remains to be tested, possibly in an axenic culture system (Baltz *et al.*, 1985).

It has also been proposed that differentiation may be controlled by changes in the intra-trypanosomal concentration of cyclic adenosine monophosphate (cAMP) (Mancini & Patton, 1981). It has been shown that slender forms near parasitaemic peak contain more cAMP than stumpy forms, and that blocking adenosine receptors (so preventing the intracellular accumulation of cAMP) inhibits differentiation (Reed *et al.*, 1985). In some eukaryotic cells intracellular increase in cAMP concentration is associated with the action of platelet-derived growth factor (PDGF) (Rozenqurt *et al.*, 1983; Rozenqurt, 1986). This is of particular interest in relation to the control of trypanosome growth since the predominance of stumpy forms at peak parasitaemia correlates with the extensive aggregation and lysis of platelets that occurs at this time (Davis *et al.*, 1974; Davis, 1982).

Receptors for transferrin and low density LDL have been shown to be present on the surface of bloodstream trypanosomes (Coppens *et al.*, 1988). These receptors are probably involved in receptor-mediated endocytosis of nutrients required by trypanosomes (Coppens *et al.*, 1987) to supply the lipid needs of the parasites. Whether these receptors have a role in the transduction of any growth-promoting

signals remains to be tested.

In summary, current research suggests that two mechanisms may control differentiation of trypanosomes in the mammalian bloodstream.

(1) Multiplication of trypanosomes, like that of many mammalian cells, is maintained by growth factors. Depletion of such growth factor(s) may trigger differentiation.

(2) Multiplication of slender form trypanosomes requires a plentiful supply of lipids from the mammalian host because these parasites lack the ability to synthesise their own. Depletion of the lipid supply may trigger differentiation.

Both of these mechanisms are reminiscent of the opinion of Robertson (1913a) that stumpy form trypanosomes are crisis forms, produced in response to adverse conditions in the host. However, trypanosomes seem to have integrated their "crises" into a highly successful lifecycle in which maximum replication can occur, followed by transmission of preadapted forms to a suitable second host species. This opinion is supported by evidence that drug treatment of trypanosomes with DL-α-difluoromethylornithine (DFMO) appears to induce production of stumpy forms (DeGee, McCann & Mansfield, 1983; DeGee *et al.*, 1984; Giffin *et al.*, 1986).

1.7 AIMS OF THE RESEARCH

The overall aim of the research presented in this thesis was to examine the mechanisms underlying pleomorphism in bloodstream trypanosome infections, and to explore the relationship between control of differentiation and the immune response in determining the shape of a parasitaemic wave. As a first step towards this aim an antigenically-defined model system of the first parasitaemic wave in a mouse was characterised. This model parasitaemia was then used to

answer three questions.

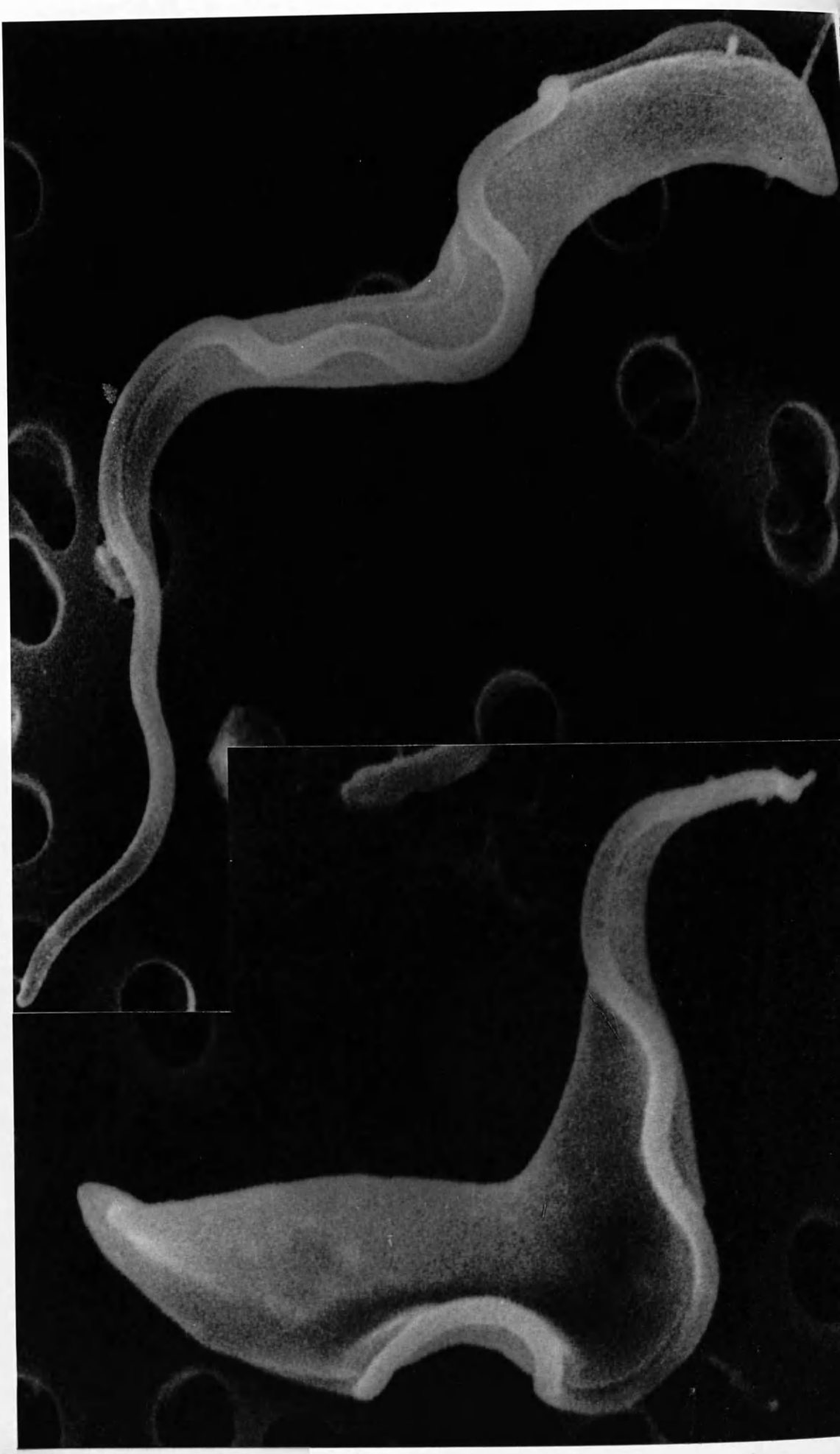
(1) Is differentiation to stumpy forms triggered by depletion of a host factor that is required for maintenance of multiplication?

(2) Is there evidence of selective killing of trypanosomes of different morphological types by immune effector mechanisms?

(3) Do bloodstream trypanosomes act as an antibody sink?

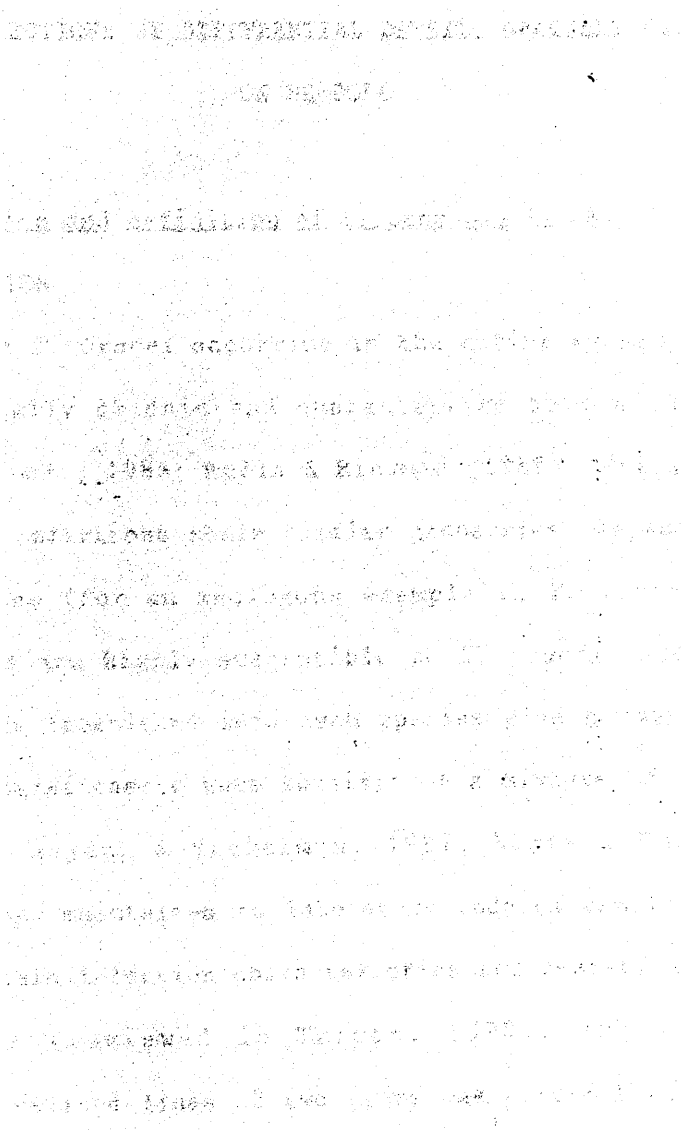
Table 1.1. Morphological features used to characterise morphological types of *T. brucei* (based on Wijers, 1959a, b). LS = long slender; LI = long intermediate; I = intermediate; SI = short intermediate; SS = short stumpy.

	LS	LI	I	SI	SS
NUCLEUS	elongated	elongated	oval	oval	round or oval
SHAPE OF POSTERIOR END	truncated	pointed	round or slightly pointed	pointed on one side	pointed on one side
POSITION OF KINETOPLAST	sub-terminal	less sub-terminal	nearly terminal	nearly terminal	nearly terminal
FREE FLAGELLUM	long	markedly shorter	same as LI	short	none
BODY WIDTH	thin	thin	thicker	thicker still	even thicker
LENGTH (μm)	29		23		18



facing page 43

Fig. 1.1. Scanning electron micrographs (x 20 000) of *T. brucei* bloodstream forms: (a) long slender form; (b) short stumpy form. (Thanks to L. Tetley for the gift of these photographs.)



Chapter 2

CHARACTERISATION OF MATERIALS:

THE DERIVATION AND DEFINITION OF TRYPANOSOME LINES

GROWTH AND MORPHOLOGICAL COMPOSITIONS OF CLONED TRYPANOSOME POPULATIONS DURING A PARASITAEMIC WAVE

ATTEMPTS TO ISOLATE PURIFIED SLENDER AND STUMPY FORM POPULATIONS FROM PLEOMORPHIC INFECTIONS BY DIFFERENTIAL DENSITY GRADIENT CENTRIFUGATION ON PERCOLL

2.1 The derivation and definition of trypanosome lines

2.1.1 INTRODUCTION

Infections with *T. brucei* occurring in the native mammalian fauna of Africa are usually chronic and characterised by low parasitaemias (Rurangirwa *et al.*, 1986; Mulla & Rickman, 1988). Primary isolates from wild type infections share similar properties, depending also on the host species (for an analogous example in *T. vivax* see Barry, 1986a). Rodents are highly susceptible to *T. brucei* infections, and primary isolates inoculated into such species give chronic infections in which each parasitaemic wave consists of a mixture of VATs (Le Ray *et al.*, 1977; Hajduk & Vickerman, 1981; Barry & Emery, 1984). Trypanosome lines maintained in laboratory rodents can be manipulated to select certain infection characteristics and thereby create useful model systems (reviewed in Turner, 1990). The use of well characterised, defined lines of two types was critical to many of the experiments described in this thesis. The first of these line types was derived by repeated passaging through rodents, with cloning and frequent recloning, eventually resulting in a line of trypanosomes

that is homogeneous with respect to VAT during the first parasitaemic peak; that is, over 95% of individuals in a population express the same VSG. Such a line still produces stumpy forms (is pleomorphic), and produces an infection that does not kill rodent hosts at the first peak (see Turner, 1990, in which these are termed "type 3" lines).

The second type of line was derived by repeated passaging of trypanosomes derived from a primary isolate through rodent hosts at 2 - 3 day intervals resulting in the formation of lines that have a very low rate of differentiation to stumpy forms and a very high degree of VAT homogeneity (Cross, 1975; Van Meirvenne, Janssens & Magnus, 1975). Such lines are extremely virulent, killing their hosts at the first peak (see Turner, 1990, in which these are termed "type 2" lines).

VAT homogeneous pleomorphic lines are valuable in research into anti-trypanosome immune responses. They allow the effects of specific immune responses to be observed in whole populations, rather than on individual trypanosomes, and the effects of the immune response are not masked by the presence of other parasites expressing heterologous VATs. VAT homogeneous monomorphic lines can be used as a model for slender forms. Another use of these lines is as a source of large numbers of trypanosomes all expressing the same VAT; 10^9 parasites/ml of blood can be obtained from rodents infected with such lines.

Before using defined lines of these types in particular experiments, detailed characterisation of three aspects of the lines was required: (1) a detailed comparative description of the growth and morphological characteristics of monomorphic and pleomorphic lines; (2) the derivation of a new pleomorphic line to which a functionally active monoclonal antibody was available; and (3) a comparison by a non-serological method of monomorphic and pleomorphic lines that

(apparently) express the same VAT. The first aspect is dealt with in section 2.2, and the other two are described below.

A technical limitation on the use of VAT homogeneous pleomorphic lines was that the examples available for use were serologically characterised using rabbit antisera and the only monoclonal antibody against a VAT expressed by such a line displayed immunofluorescent labelling activity but no functional activity. To overcome this limitation, I have derived a new pleomorphic line that homogeneously expresses a VAT to which a functionally active monoclonal antibody was already available. The VAT expressed by this line is GUTat 7.2.

In the experiments described in this thesis two pairs of serologically identical trypanosome lines were used. One of each pair was monomorphic and the other was pleomorphic. The antigenic identity of the two parasite lines within each pair was determined using rabbit antisera and/or mouse monoclonal antibodies. However, cross reactions of monoclonal antibodies and specific antisera with two VATs within the same serodeme has been reported (Barbet, Davis & McGuire, 1982; Crowe, 1983). This was attributed to the presence of shared epitopes on different VSG molecules. To ensure, therefore, that each pair of lines does indeed express the same VAT, I have compared them using a non-serological method. To determine if there is a physico-chemical difference between the VSG molecules on (apparently) serologically identical trypanosomes, the isoelectric points (pIs) of the four VATs were compared by isoelectric focusing (IEF). This technique was chosen because it is particularly sensitive at detecting minor differences in amino acid sequence between VSGs. VSG molecules derived from different trypanosome isolates which were serologically cross-reactive, and had a large degree of amino acid sequence homology had different pIs

(Vervoort *et al.*, 1981). Similarly, Roelants *et al.* (1985) showed that serologically identical VSG molecules varied in molecular weight and pI.

2.1.2 DEVELOPMENT OF A PLEOMORPHIC LINE EXPRESSING GUTat 7.2

2.1.2.1 Optical cloning

Trypanosomes were cloned optically using the following method. A drop of blood from a mouse infected from stabilate GUP 2898 was diluted in normal guinea pig serum, and a fine pin was used to spot a small drop of this mixture into each well of a "Terasaki" tissue culture plate (0.01 ml working volume). These very small drops evaporate very quickly, so the interior of the plate was kept humid by the inclusion of damp tissue, and the procedure was carried out in a cool room. The drops of serum were examined using an inverted microscope: any wells containing a single trypanosome were immediately filled with phosphate buffered saline including 1% glucose (PSG) to prevent evaporation of the drop and death of the trypanosome. Every trypanosome-containing drop was injected into the peritoneum of a separate immunosuppressed mouse. After approximately 6 days 2 out of 10 mice displayed a parasitaemia, and blood smears taken from these were tested by immunofluorescence using an antibody specific for GUTat 7.2. A second cloning procedure was carried out on a parasite population containing a significant proportion (45%) of GUTat 7.2, and a population expressing 100% GUTat 7.2 was obtained. This was made into the stabilate GUP 2909.

2.1.2.2 Immunofluorescence reactions

The antigenic identities of the parasite populations derived from cloned trypanosomes were determined on thin blood smears by an indirect immunofluorescence test (indirect IFT; Turner, Barry &

* Antisera specific for heterologous antigen types were used as negative controls in these and all other immunofluorescence experiments.

Vickerman, 1988, based on the method of Van Meirvenne, Janssens & Magnus, 1975). The smears were air-dried and fixed in acetone for 15 min. Either a GUTat 7.2-specific rabbit antiserum (at 1/100 dilution) or the undiluted supernatant of a monoclonal antibody-producing hybridoma cell culture specific for GUTat 7.2 (Turner, Barry and Vickerman, 1986) was applied to the slides as the first antibody. Fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse immunoglobulin (Scottish Antibody Production Unit) at 1/50 dilution in PBS containing propidium iodide (1/10000) was used as the second antibody.*

2.1.2.3 Results

A new pleomorphic line that stably expresses GUTat 7.2 was derived by cloning from a relapse population of GUTat 7.13 and recloning twice (Fig.2.1). This new line has similar growth and morphological characteristics to the pleomorphic GUTat 7.13 from which it was derived.

2.1.3 COMPARISON OF VSG MOLECULES BY ISOELECTRIC FOCUSING

2.1.3.1 Trypanosomes

Three lines were derived from the cloned stock isolate EATRO (East African Trypanosomiasis Research Organization) 2340. One of these, GUTat (Glasgow University *Trypanozoon* antigen type) 7.2, is monomorphic (Barry, Crowe and Vickerman, 1985) and a second, GUTat 7.13, is pleomorphic (Turner, Barry and Vickerman, 1986). A pleomorphic line that stably expresses GUTat 7.2 was derived from a relapse population of GUTat 7.13 (described in section 2.1.2). The fourth parasite line was derived from a cloned stock EATRO 795 (Turner & Barry, 1989). It was designated ILTat (ILRAD *Trypanozoon* antigen type) 1.64, is monomorphic, and is serologically identical to GUTat

2.1.3.2 Separation of trypanosomes from blood

The anion-exchange column method of Lanham (1968) was used. Briefly, trypanosomes are less negatively charged than red blood corpuscles (RBC). The RBC are detained by adherence to the positively-charged diethylaminoethyl sidechains conjugated to cellulose (DEAE-cellulose) while the trypanosomes are eluted. The degree of adsorption of RBC to the DEAE-cellulose increases with their negative charge. The most important factors are the ionic strength and pH of the eluting buffer. Using PSG (I=0.217, pH 8) excellent separation of *T. brucei* from rodent blood is achieved (Lanham & Godfrey, 1970).

For the purification of VSG large numbers of trypanosomes were obtained from the blood of several female CFLP mice or male Wistar rats on days 3 or 4 post-infection when the parasitaemias were approximately 5 x 10^8 trypanosomes/ml of peripheral blood. The blood was mixed with half its volume of PSG (pH 8) containing 0.2mM proteinase inhibitors N-ethylmaleimide (NEM), Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) and phenylmethyl sulfonyl fluoride (PMSF). This was to prevent proteolytic cleavage of the VSG molecules from the surfaces of the trypanosomes before the parasites were purified from the other blood components. This pH 8 buffer was also used to elute purified parasites from the DEAE-cellulose anion-exchange column as previously described.

2.1.3.3 Isolation and purification of VSG

Parasites eluted from a DEAE-cellulose column were washed in PSG (pH 8) and resuspended in a small volume (approximately 5 ml) of 0.125M PBS (pH 5.5) which also contained 1% glucose and 0.2mM proteinase inhibitors NEM, TLCK and PMSF. This suspension of parasites was

incubated overnight at 4°C during which time VSG was cleaved from the parasites surfaces. The suspension was centrifuged at 1000 x g for 10 mins to pellet out the trypanosomes and the supernatant which contained the VSG was ultracentrifuged (30 000 rpm, 10 x 10 rotor head, 60 mins, 4°C). This method was based on that of Huet-Duvillier *et al.* (1988), in which increasing amounts of released VSG monomers were formed with time during the pH 5.5 incubation. The samples were freeze-dried and stored at -40°C.

2.1.3.4 Isoelectric focusing of VSGs

IEF was conducted using equipment and chemicals from Pharmacia unless otherwise indicated, and using the manufacturers recommended procedures. Agarose gels (1% w/v) were made using D-sorbitol (gold label grade, Aldrich) and ampholytes (pH 3-10 range). The gel was poured onto a plastic substrate (Gelbond) and set overnight at room temperature. To run the gel, electrodes were soaked in (0.01M H_2SO_4 for the positive electrode and 0.1M NaOH for the negative electrode, 25 μl VSG samples (approximately 10 mg of protein/ml) and pI markers applied. Mouse blood was also applied to a spare track, the haemoglobin was a useful visible marker of focusing. The apparatus was run at 1000 V, 20 mA, 7.5 W, with cooling until the haemoglobin had focus ed into three sharp bands (approximately 60 mins). The gel was fixed, dried, and stained 0.25% Coomassie blue. The pIs of the VSG samples were estimated by comparison with the positions of the marker bands of known pI.

2.1.3.5 Results

The purified VSGs from both monomorphic and pleomorphic GUTat 7.2 gave a doublet of bands on agarose-IEF gels. The patterns obtained with both VATs were indistinguishable from each other. The pI value of the denser band was 6.5 and the lesser band had a slightly lower pI (6.0 <

pI < 6.5). The pI value obtained for VSG samples derived from monomorphic ILTat 1.64 and pleomorphic GUTat 7.13 was 6.0. In the case of these two VATs only a single band was present. The gels obtained in this experiment were not of publication quality and, therefore, have not been included.

2.1.3.6 Discussion

The patterns of the bands were very similar within each VAT pair, but different between the pairs. These results confirm and extend the serological observations that GUTat 7.2 in the two parasite lines is the same, and that GUTat 7.13 is the same as ILTat 1.64. The similarity in the pI values of the VSGs derived from monomorphic and pleomorphic lines expressing GUTat 7.2 is not surprising. Both lines were derived from stock EATRO 2340, and would be expected to be transcribing from the same VSG gene, of which there is only one copy in this stock (Cornelissen *et al.*, 1984). The similarity in pI values for the VSGs derived from the lines expressing ILTat 1.64 and GUTat 7.13 is more surprising because these lines are derivatives of different original isolates. ILTat 1.64 was derived from an original isolate EATRO 795 from a wild African ungulate in 1964 in Kenya (Onyango, Van Hoeve & De Raadt, 1966), GUTat 7.13 was derived from EATRO 2340 from a Kenyan human in 1977. These two stocks belong to the same population (Barry *et al.*, 1983; Tait *et al.*, 1985) and thus the two VATs can be considered as samples that differ by 13 years in time. This similarity in the VSG molecules on two separate parasite isolates seems to indicate conservation of VSG genes during evolution. This strongly contrasts with findings of changes in the VAT repertoire during the same time period in this population (Barry, Crowe & Vickerman, 1983), i.e. trypanosomes show a high rate of change in VAT repertoires but a negligible change in any one of the constituent VATs. This is

understandable in that a completely new VAT, serologically unrelated to any others, can be useful in extending the duration of an infection. Point mutations in a VSG gene, however, will lead to the production of a modified VAT that is highly likely to serologically cross-react with its predecessor and can therefore make no functional contribution to the course of an infection. Thus there is selective pressure at the level of the individual VAT for conservation. For the observed changes (Barry, Crowe & Vickerman, 1983) in VAT repertoires to occur in trypanosome populations, large structural changes in VSG genes, or activation of previously inactive VSG genes must occur (Bernards *et al.*, 1986).

An additional method of observing similarities (or differences) in the VSG molecules derived from iso-VATs is to digest samples of the VSG with staphylococcal V8 protease or cyanogen bromide and examine the patterns of digestion products on SDS-PAGE (Newbold *et al.*, 1984; Miller, Allan & Turner, 1984).

2.2 Growth and morphological composition of cloned trypanosome populations during a parasitaemic wave

2.2.1 INTRODUCTION

The general properties of parasitaemia curves have already been described for monomorphic (Ashcroft, 1960) and pleomorphic (Ashcroft, 1957; Balber, 1972) trypanosome lines. Those studies, however, used uncloned and uncharacterised trypanosome isolates. I have studied the growth and morphological characteristics of the pleomorphic line GUTat 7.13 and a monomorphic line, ILTat 1.64. These two lines were selected for this characterisation because their VSGs are identical, both serologically and with respect to pI. These observations form a sound basis upon which subsequent experiments to determine the relationship

Except where otherwise stated, all mice were adult (2-5 month old) females. For all syringe inoculations trypanosome-infected blood was diluted in Hanks buffered saline (pH 7.2) containing heparin (10-20 units /ml).

of trypanosome pleomorphism to host immune responses could be based.

2.2.2 MATERIALS AND METHODS

2.2.2.1 Trypanosomes

The parasitaemia curves and morphological characteristics of two cloned VAT homogeneous trypanosomes lines were studied. The lines were pleomorphic GUTat 7.13 (Turner, Barry & Vickerman, 1986) and monomorphic ILTat 1.64 (Turner & Barry, 1989).

2.2.2.2 Estimation of proportions of morphological forms

Bloodstream trypanosomes were categorised as slender (including dividing forms), intermediate or stumpy based on the method of Wijers (1959a). Bloodsmears were air-dried, fixed in methanol for 2 mins and Giemsa-stained for 10 mins. The slides were then washed in dd H_2O, air-dried and examined by light microscope at x400 and x1000 (oil-immersion).

2.2.2.3 Prevalences of morphological variants: infections with pleomorphic GUTat 7.13

A group of six female BALB/c mice were inoculated intraperitoneally with pleomorphic GUTat 7.13 from stabilate. From day 4 (when the parasitaemia became patent) onwards accurate counts of the number of trypanosomes present per ml of blood were made twice daily as follows. A measured amount of tail blood was diluted into a known volume of 0.85% NH_4Cl and the number of trypanosomes present was counted in an improved Neubauer haemocytometer. Geometric mean parasitaemia ± 2 SE was calculated for the six mice at each time-point. At each time-point blood smears were made, and Giemsa-stained to determine the relative abundances of the different morphological types. Arithmetic mean percentage ± 2SE was calculated for the six mice at each time point. To determine the absolute abundance of each morphological type the

proportions of each type were multiplied by the absolute abundance estimate for each mouse. These results are expressed as geometric mean abundance ± 2SE.

2.2.2.4 Prevalences of morphological variants: infections with pleomorphic GUTat 7.13 or monomorphic ILTat 1.64 in immunocompetent and immunosuppressed mice

The numbers of the different morphological forms present during the course of infections with pleomorphic and monomorphic parasites were compared in immunocompetent and immunosuppressed mice. The mice in one group were chemically immunosuppressed 24 h before infection with cyclophosphamide (250 mg/kg).

Infection with pleomorphic GUTat 7.13

Two groups of female BALB/c mice were inoculated intraperitoneally with 10^5 parasites/mouse. The mice in one group were given cyclophosphamide 24 h before infection. Accurate counts were made as above of the numbers of parasites present/ml of blood in mice in both groups, and smears were made for differential morphological counting.

Infections with monomorphic ILTat 1.64

As above except that mice were inoculated intravenously.

2.2.3 RESULTS

2.2.3.1 Prevalences of morphological variants: infection with pleomorphic GUTat 7.13

The changes in relative proportions of slender, intermediate and stumpy forms with time during the first parasitaemic wave of infection are shown in Fig. 2.2a. The total numbers of parasites and the numbers of each of the morphological categories present at each time point are shown in Fig. 2.2b. For clarity the figures show the mean results only. These results are also given in Table 2.1 as means ± 2 SE.

The total parasitaemia increased most rapidly during the first 5 days of infection. During this period the slender forms predominated (Fig. 2.2a): over 75% of parasites were slender (or dividing) on day 3 post-infection, although the numbers of all morphological types, not just slender forms, were increasing (Fig. 2.2b). The relative proportions of the morphological types varied as the infection proceeded (Fig. 2.2a): the proportion of intermediate forms increased from day 3 onwards, followed within 24 h by an increase in the proportion of stumpy forms. This correlated with a decrease in the growth rate observed in the plot of total parasitaemia between days 4 and 5. By days 5-6 the graph of total parasitaemia had reached a plateau phase after which the parasitaemia began to decline. At this time the proportion of stumpy forms was at its highest (57%) and the proportion of slender forms was at its lowest (6%). Although the stumpy forms predominated over the slender forms at this point in the infection, they never achieved the same high proportion that the slender forms did during the rising phase of the parasitaemia.

2.2.3.2 Prevalences of morphological variants: infections with pleomorphic GUTat 7.13 or monomorphic ILTat 1.64 in immunocompetent and immunosuppressed mice

Infection with pleomorphic GUTat 7.13: immunocompetent mice

This was, in effect, a repeat of the experiment described in the previous section. It is included here because it was conducted in parallel with the following three experiments and because a comparison of the results presented in Figs 2.2 and 2.3 illustrates the degree to which variability is encountered in parasitaemias of this line of trypanosomes.

In mice with normal immune function infected with pleomorphic GUTat

7.13 the parasitaemia peaked on day 4 post-infection and then declined sharply (Fig. 2.3a). The proportion of slender forms which was 89% at t=72 h (day 3) declined sharply to 5% on day 5 and then rose again (Fig. 2.3b). The proportion of intermediate forms peaked on day 4 at 64%, declined to 19% by day 6. The curve representing the proportion of stumpy forms echoed the curve for the intermediate forms, peaking on day 5 at 59% and then dropping to about 40% on day 6.

Infection with pleomorphic GUTat 7.13: immunosuppressed mice

In mice infected with GUTat 7.13 in which immune function had been suppressed the parasitaemia peaked between days 4 and 5 and stayed high (Fig. 2.4a). By day 5 the initially high proportion of slender forms (83%) had declined to 20%, and remained at this level (Fig. 2.4b). The proportion of intermediate forms peaked on day 4 at 47% and oscillated about 43% from days 4-6. The proportion of stumpy forms reached a plateau at about 35% between days 4 and 6.

Infections with monomorphic ILTat 1.64

The use of intravenous inoculation reduced the prepatent period. Immunosuppression before infection had no effect on the outcome of monomorphic infection. Mice in both groups had > 10^9 parasites/ml of blood by day 4 post-infection (Fig. 2.5) and they were humanely killed at this time. Stumpy forms were not detected at any stage.

2.2.4 DISCUSSION

My experiments using the cloned and antigenically stable trypanosome line GUTat 7.13 (Figs 2.2 and 2.3) confirm and extend the findings of Balber (1972) which were obtained using an uncloned and antigenically uncharacterised line. In the experiments described some mice were pharmacologically immunosuppressed with cyclophosphamide. This drug interferes with cell division so that it prevents the rapid

proliferative responses of lymphocytes in response to antigen. Cyclophosphamide is not specific for lymphoid cells and cell division in other rapidly dividing cells, such as trypanosomes and in gut epithelia, is also adversely affected, making this a toxic drug to use. However, the half-life of the drug is relatively short so that the division of trypanosomes inoculated into mice 24 h after treatment with cyclophosphamide is not adversely affected (Bach & Strom, 1985).

These results provide accurate baseline information for a pleomorphic, homogeneous infection on which subsequent experiments have been based. The most important concept in understanding the model parasitaemic peak is the distinction between the proportion of a given morphological type present in the trypanosome population, and the total number of parasites with that morphology in the population. In immunologically intact mice the absolute number of slender forms peaked on day 4 and thereafter declined sharply (Fig. 2.2b), whereas the absolute number of stumpy forms continued to rise until day 5. The change in predominance from slender to stumpy forms (Fig. 2.2a) correlated with the sudden and rapid reduction in the number of slender forms just after the parasitaemic peak, rather than with an absolute increase in the number of stumpy forms.

The proportions of stumpy forms increased rapidly during the first few days of infection with pleomorphic GUTat 7.13 in both immunocompetent mice (Fig. 2.3b) and cyclophosphamide-treated (immunosuppressed) mice (Fig.2.4b), and stumpy forms were produced throughout infections in immunosuppressed mice. These facts are incompatible with theories that differentiation to stumpy forms is triggered by the effect of specific antibody (Ashcroft, 1957; Wijers, 1957, 1959b) or a putative promoter of differentiation. Balber (1972) speculated that the host antibody

response may alter the morphological composition of the peripheral blood population without influencing the transformation of the slender forms into stumpy forms, perhaps because of differential sensitivity to antibody. The role of specific antibody in clearance of trypanosomes from the bloodstream is explored in Chapter 4.

2.3 Attempts to isolate purified slender and stumpy forms from pleomorphic populations

2.3.1 INTRODUCTION

The most useful way to investigate the relationship between pleomorphism of African trypanosomes and mammalian control of the infection would be to study each component separately. That is to say, the control, killing and clearance (or otherwise) of slender form trypanosomes should be studied in isolation from the stumpy forms and vice versa. This is possible to a limited extent because lines of African trypanosomes that are continuously passaged through laboratory rodents, without any episodes of cyclical transmission through tsetse flies lose their ability to differentiate into stumpy forms. Such lines develop increased virulence: slender forms divide causing extremely high parasitaemias with relatively faster population doubling times. The presence of huge numbers of parasites in the circulation leads to death of the host before the immune response can become effective against the dominant antigen type(s). These changes provide a model of slender form parasites, although the validity of monomorphic trypanosomes as a model for slender forms has been established only in terms of gross morphology and lack of mitochondrial activation. Even if monomorphic trypanosomes are an acceptable model for slender form trypanosomes, no equivalent model exists for stumpy form bloodstream trypanosomes.

An alternative strategy that can be used to compare slender and stumpy forms involves the isolation of predominantly slender populations from the rising phase of a parasitaemic wave and predominantly stumpy populations from the plateau phase (see section 2.2) (Black *et al.*, 1983). The analysis of the morphological composition of bloodstream infections with GUTat 7.13 in section 2.2 showed that when the parasitaemia became patent and for the following 24-48 h the parasite population was composed predominantly of slender forms. Thereafter, as the parasitaemia approached its peak the relative proportion of stumpy forms increased. Thus there was a natural enrichment for slender forms early in the parasitaemia and a relatively small degree of enrichment for stumpy forms later as the parasitaemia passed its peak value and began to decline. The use of parasite lines which are pleomorphic but relatively antigenically stable means that the predominantly stumpy form populations obtained just after the parasitaemic peak are still expressing the original antigen type. The reservation with this strategy, however, is that populations from different time-points in an infection may vary in parameters other than their morphology.

A method was sought, therefore, for the separation of viable slender or stumpy forms from the same bloodstream population. Some preliminary experiments by C.M.R. Turner (described in section 2.3.2) suggested that differential density centrifugation using Percoll might allow the separation of subpopulations enriched for each of the two morphological types. The buoyant density of trypanosomes was found to be approximately 1.07 g/ml, so gradients with average values around 1.07 should give maximum possible resolution between different subpopulations of trypanosomes. To develop this method of separation of morphological types of trypanosomes, the behaviour of predominantly slender and predominantly stumpy trypanosome populations were compared

in both preformed and *in situ* forming Percoll gradients.

As the name suggests, separation on *in situ* formed Percoll gradients involves mixing the cells with the Percoll solution followed by centrifugation so that gradient formation and cell separation occur simultaneously. Obviously, the centrifugal force that can be applied to the gradients is limited, being dependent on the ability of the cells being separated to withstand the pressure.

Preformed gradients were created by very high-speed (at least 20 000 x g) centrifugation. The profile of the resulting gradient is dependent on the centrifugal force applied, angle of the rotor-head, size of centrifuge tube, diluent used, and length of time. Therefore, before attempting to use Percoll gradients for separation of trypanosome populations, a series of standard curves was produced using various concentrations of Percoll and density marker beads. These curves characterised the gradients produced by the apparatus used in the subsequent experiments.

Preformed gradients were then used to separate cells (which would not survive large centrifugal forces) at relatively low speeds: the cells sedimented to positions in the gradient where the density equalled their own. Preformed gradients can be stored for several weeks, therefore many gradients were prepared at the same time to enhance reproducibility between experiments.

2.3.2 PRELIMINARY EXPERIMENT TO TEST THE FEASIBILITY OF SEPARATING MORPHOLOGICAL FORMS OF *T. BRUCEI* BY DIFFERENTIAL DENSITY CENTRIFUGATION ON PERCOLL GRADIENTS FORMED *IN SITU*

This section describes a preliminary experiment carried out by C.M.R. Turner. Blood was obtained from female CFLP mice infected with GUTat 7.13 on days 3 and 6 post-infection. Trypanosomes were then crudely

purified by centrifugation at 2000 x g for 10 mins, and the trypanosome-containing cell layer between the RBC and serum removed. The trypanosomes were then washed and resuspended in PSG. The yield of slender forms (day 3) was 7.8 x 10^6 trypanosomes/100 μl of PSG, and the yield of stumpy forms (day 6) was 9.15 x 10^6 trypanosomes/ 100 μl of PSG. Nine sets of triplicate eppendorf tubes were prepared containing various ratios of stock isosmotic Percoll and Hank's balanced salt solution in the range 860 μl:240 μl to 700 μl:400 μl. The first tube of each set contained 100 μl of density marker bead suspension, the second contained 9.15 x 10^6 stumpy form trypanosomes in 100 μl of PSG, and the third contained 7.8 x 10^6 slender form trypanosomes in 100 μl of PSG. The tubes were centrifuged at 3000 x g for 2 mins. The positions of trypanosome bands in the tubes were recorded and compared to each other and to the positions of the density marker beads. The apparent buoyant density of the slender form trypanosome population was 1.087 g/ml, and of the stumpy form population was 1.064 g/ml. The best segregation of these two populations was achieved in two tubes that had SIP:saline ratios of 740 μl:360 μl and 720 μl:380 μl. These results are illustrated in Fig. 2.6.

2.3.3 MATERIALS AND METHODS

A solution of stock isosmotic Percoll (SIP; 280-320 mOsm/kg H_2O) was made up of 9 parts Percoll plus 1 part 1.5M NaCl. The SIP was used in the experiments that follow to make density gradients with various average density values and of different types. This was achieved by diluting the SIP in 0.15 M NaCl.

2.3.3.1 Separation of slender and stumpy populations on Percoll gradients formed *in situ*

Mixtures of SIP and 0.15M NaCl in various relative proportions (Table 2.2) were prepared in triplicate in eppendorf microcentrifuge tubes. Trypanosomes (approximately 10^7/100 μl) taken from mice on days 3 or 6 after infection with pleomorphic GUTat 7.13, or density marker beads in 100 μl suspension were layered on top of the mixture and the tubes were centrifuged at 3000 x g for 2-5 mins in a benchtop microcentrifuge.

2.3.3.2 Separation of slender and stumpy populations on preformed Percoll gradients

A preliminary experiment was conducted to determine the relationship of SIP concentration and density gradient profile. A series of 8 ml tubes with various concentrations of SIP in them were prepared. A suspension of colour-coded density marker beads was added to all tubes before these were centrifuged (20 000 rpm, 30 mins, 4°C) in an angle-headed rotor (23°). The marker beads have different buoyant densities and trace the density gradient in the centrifuge tubes.

Three identical Percoll gradients giving wide separation in the density range 1.064 - 1.087 g/ml were prepared. SIP and 0.15 M NaCl were mixed in the ratio 3:2, and the gradients were preformed by ultracentrifugation (23° rotor head, 20 000 rpm, 30 mins, 4°C). Infected blood from female CFLP mice infected with GUTat 7.13 was taken by cardiac puncture on days 4 and 6 post-infection and centrifuged at 1000 x g for 10 mins. The trypanosome-containing layer of cells at the interface between RBC and serum was removed, washed and resuspended in PSG to give a suspension of approximately 10^8 parasites in 0.5 ml. Samples were layered onto the preformed gradients as follows. Gradient 1 had 0.5 ml of the suspension of day 4 (slender

form) trypanosomes layered onto it, 0.5 ml of the day 6 (stumpy form containing) suspension was layered onto gradient 3, and 0.5 ml of PSG containing density marker beads was layered onto gradient 2. These were centrifuged at 1000 x g for 10 mins. After centrifugation the positions of the trypanosome and marker bead bands were measured and plotted to give a curve representing the density gradient. Then the trypanosome-containing bands were removed, washed in PSG to remove Percoll, resuspended in PSG and smeared onto microscope slides coated with poly-L-lysine. These smears were Giemsa stained as described previously and stored for differential morphological counts.

A repeat of this experiment was conducted as follows. The same gradient mixture described above was used to differentiate between monomorphic GUTat 7.1 parasites (representing slender forms) and day 6 pleomorphic GUTat 7.13 (predominantly stumpy form) parasites. Samples were layered onto the preformed gradients as follows. Gradient 1 had 0.5 ml containing 0.5×10^8 monomorphic GUTat 7.1 (slender form) trypanosomes layered onto it, 0.5 ml (containing 10^8 stumpy form parasites) of the day 6 GUTat 7.13 trypanosome suspension was layered onto gradient 3, and 0.5 ml of PSG containing density marker beads was layered onto gradient 2. These were centrifuged at 1500 x g for 20 mins. After centrifugation the positions of the trypanosome and marker bead bands were measured and plotted to give a curve representing the density gradient.

2.3.4 RESULTS

2.3.4.1 Separation of slender and stumpy populations on Percoll gradients formed *in situ*

In tubes 1 - 6 all parasites were localised to the top portion of the gradient and in tubes 9 - 18 the reverse was true (Table 2.2). (Tubes

8, 10 and 12 have not been included in Table 2.2 because insufficient numbers of trypanosomes were used and no parasite-containing bands were visible in these tubes.) The density gradient that gave separation of slender and stumpy trypanosomes (tube 7) had SIP and saline in the ratio 3:2. A comparison of the results for duplicates of some gradients (tubes 5, 7 and 9) shows that there was poor reproducibility.

2.3.4.2 Separation of slender and stumpy populations on preformed Percoll gradients

Density marker beads were used to describe the profile of the gradients obtained with SIP and 0.15 M NaCl in several ratios in our particular centrifugation conditions. The distance from the bottom of the centrifuge tube of each type of bead was plotted against the density of the beads. The gradient around the target density of p = 1.064 - 1.087 g/ml was shallowest in the tube containing SIP and saline in the ratio 3:2 (Fig. 2.7). Separation of trypanosome-containing bands should be greatest, therefore, on preformed gradients containing SIP and saline in the ratio 3:2.

In subsequent experiments gradients with SIP and saline in the ratio 3:2 were used. The gradient profile obtained in one experiment is shown in Fig. 2.8. The slender form trypanosomes settled 16-21 mm from the bottom of the tube. This corresponded to a buoyant density of 1.070-1.101 g/ml. The stumpy form parasites settled 19-22.5 mm from the bottom of the tube, corresponding to a buoyant density of 1.069-1.090 g/ml, i.e. higher than the preliminary experiment had suggested. Smears were made of samples taken from the top and bottom of the parasite-containing bands and Giemsa-stained for differential morphological counts (Table 2.3). The top of the band in the tube that

contained day 6 parasites was composed entirely of stumpy forms, intermediate forms having settled at the bottom of the band. The tube containing day 4 parasites produced a poor separation of slender forms throughout the parasite-containing band.

2.3.5 DISCUSSION

In a preliminary experiment conducted by C.M.R. Turner, day 3 (slender) and day 6 (stumpy) subpopulations of *T. brucei* migrated to different bands in 1.2 ml Percoll gradients with average density 1.07 g/ml formed *in situ* by centrifugation at 3000 x g for approximately 5 mins (section 2.3.2). The result was not reliably reproducible; replicate experiments showed extensive overlap of bands containing slender and stumpy forms, and the parasite-containing bands were very diffuse.

Attempts were made to use larger volume preformed gradients in 8 ml tubes to obtain greater separation of larger numbers of trypanosomes. Again results were variable, with overlap of bands, and very diffuse bands being common. Overlap of the positions of bands of slender and stumpy forms made removal of the two populations difficult. Furthermore, several washing stages were required to remove most of the Percoll from the parasite preparations, and excessive washing caused vacuolation of the trypanosomes which is probably indicative of reduced cell viability. A possibility that was not tried was to make several stock solutions of different densities which can be layered one below another to give a step gradient through which a cell suspension can be spun.

There are three main characteristics of trypanosomes that make them unsuitable for differential density centrifugation. The first is their elongated shape. The behaviour of near spherical cells on Percoll

gradients can be directly related to their density. Percoll itself is a suspension of spherical beads which move relative to each other in a defined manner during centrifugation. Elongated cells probably have different rates of movement through Percoll depending upon their orientation. This means that precisely-defined bands corresponding to different densities may not necessarily be obtained. The second property of trypanosomes that prevents formation of tight bands is their high degree of motility. Thirdly, bloodstream trypanosomes are present in a spectrum of morphological forms ranging from slender to stumpy and not just in these two forms.

In light of these experimental results, no further attempts to separate slender and stumpy forms trypanosomes on Percoll gradients were made, as this procedure seemed unlikely to produce materials of quality superior to those obtainable by other means. Highly pure slender forms (>95%) can be obtained early in infection (before day 3) and monomorphic lines can be used; therefore there is no advantage to using Percoll for enrichment of slender forms. For purification of stumpy forms, which only reach prevalences of about 70% in the bloodstream, and then are contaminated by slender forms expressing heterologous antigen types, differential density centrifugation on Percoll could be a useful method. However, populations enriched for stumpy forms could also be obtained from immunosuppressed animals on day 7 post-infection (see Figs 2.3a and 2.3b).

Table 2.1. Changes in the relative abundance (a) and absolute abundances (b) of different morphological types of GUTat 7.13 during the first parasitaemic wave of infection. Results are expressed in (a) as arithmetic mean percentage ± 2 SE, and in (b) as $\log_{10}$ geometric mean ± 2 SE.

(a)

TIME (h)	SLENDER FORMS	INTERMEDIATE FORMS	STUMPY FORMS
93	75.2 ± 6.3	24.0 ± 7.5	0.9 ± 1.8
100	72.8 ± 11.9	25.8 ± 12.9	1.4 ± 1.3
115	72.4 ± 15.5	21.0 ± 10.7	7.1 ± 6.6
124	45.7 ± 28.1	35.5 ± 9.1	18.8 ± 20.6
139	17.8 ± 9.4	48.6 ± 16.5	31.0 ± 23.9
148	12.5 ± 9.0	44.7 ± 9.7	42.8 ± 16.9
163	6.2 ± 3.6	36.6 ± 17.2	57.2 ± 17.5

(b)

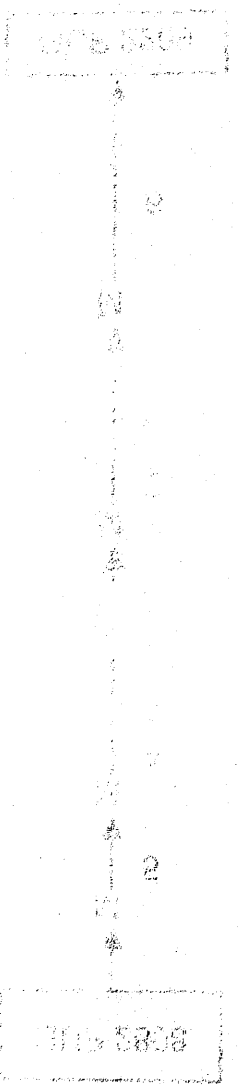
TIME (h)	TOTAL PARASITAEMIA	SLENDER FORMS	INTERMEDIATE FORMS	STUMPY FORMS
93	6.4 ± 0.4	6.2 ± 0.4	5.7 ± 0.4	-
100	6.9 ± 0.1	6.8 ± 0.8	6.2 ± 0.2	5.4 ± 0.2
115	7.8 ± 0.3	7.6 ± 0.3	7.0 ± 0.4	6.8 ± 0.6
124	8.2 ± 0.1	7.8 ± 0.4	7.7 ± 0.2	7.2 ± 0.6
139	8.2 ± 0.2	7.4 ± 0.4	7.8 ± 0.3	7.2 ± 0.9
148	7.9 ± 0.3	6.9 ± 0.5	7.4 ± 0.4	7.5 ± 0.1
163	7.4 ± 0.7	6.3 ± 0.8	6.9 ± 0.9	7.1 ± 0.6

Table 2.2. Separation of slender and stumpy forms on Percoll gradients formed *in situ*. The total volume of SIP plus saline in each tube was 1.1 ml, and 100 μl of trypanosome or density marker bead suspension was layered on top of this mixture. The results from three gradients containing SIP and saline in the ratio 700:400, and from two gradients each of ratios 690:410 and 680:420 are included to demonstrate the lack of reproducibility inherent in this technique.

TUBE	SIP:saline	DISTANCE FROM BOTTOM OF TUBE (mm)	
		SLENDER FORMS	STUMPY FORMS
1	780:320	23.5-25.0	25.5
2	760:340	24.0	23.0-24.0
3	740:360	21.0-24.0	25.0-26.0
4	720:380	22.0-24.0	25.0
5	700:400	19.0-24.0	20.0-23.0
		25.0	19.0-22.0
		20.0-25.0	26.0
6	695:405	15.0-26.0	-
7	690:410	-	19.0-26.0
		15.0-25.0	-
9	680:420	15.0	19.0-26.0
		-	2.0- 6.0
11	670:430	2.0-10.0	-
13	650:450	2.0- 7.0	2.0- 9.0
14	640:460	2.0- 7.0	6.0- 9.0
15	630:470	-	4.0- 7.0
16	620:480	-	4.0- 6.0
17	600:500	24.0	2.0- 6.0
18	550:550	1.0- 6.0	2.0- 5.0

Table 2.3. Differential morphological counts of parasite containing bands obtained by centrifugation of predominantly slender (day 4) and predominantly stumpy (day 6) parasites expressing GUTat 7.13 on a preformed Percoll gradient with SIP and saline in the ratio 3:2 (see also Fig. 2.8).
The parasite-containing bands were very diffuse. Smears made from samples taken from the top and bottom of each band were fixed, stained and analysed (see section 2.3.4.2).

PARASITES ISOLATED ON DAY	POSITION IN BAND	% SLENDER FORMS	% INTERMEDIATE FORMS	% STUMPY FORMS
4	TOP	72.2	0	27.8
4	BOTTOM	62.1	0	37.9
6	TOP	0	0	100.0
6	BOTTOM	21.4	35.7	42.9



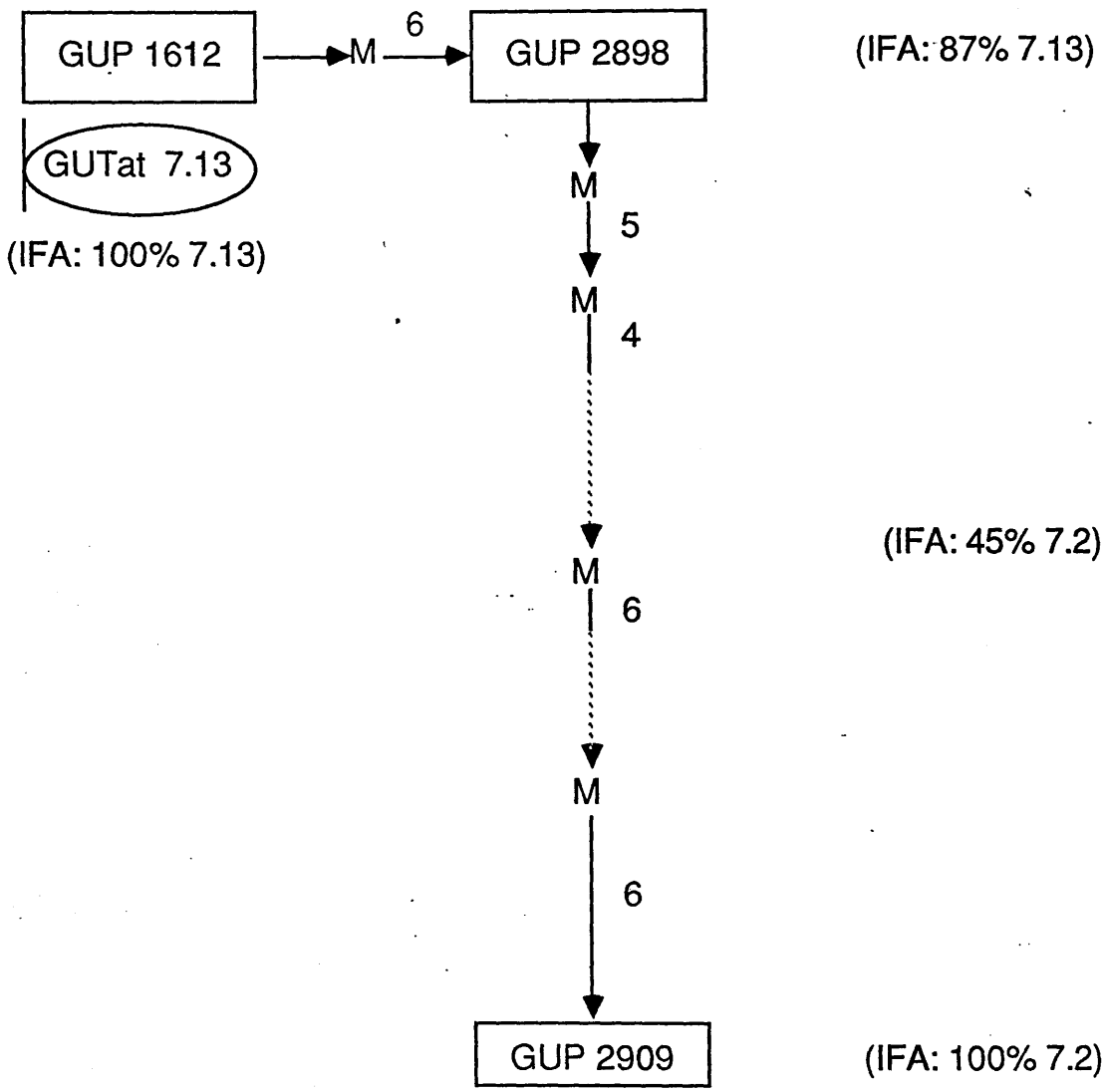
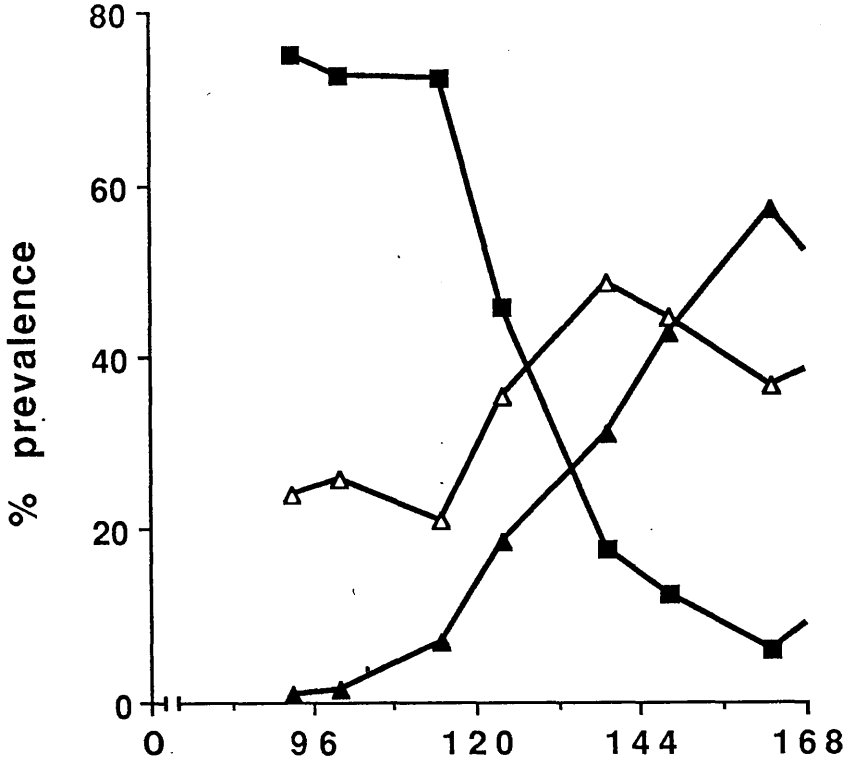

GUP 1612
M
6
GUP 2898
(IFA: 87% 7.13)
GUTat 7.13
(IFA: 100% 7.13)
M
5
M
4
M
6
(IFA: 45% 7.2)
M
6
GUP 2909
(IFA: 100% 7.2)

Fig. 2.1. Pedigree diagram of pleomorphic GUTat 7.2.

This pedigree is drawn according to the conventions of Lumsden, Herbert & McNeillage (1973). Stabilate numbers are in boxes; VAT reference stabilate 7.13 is shown in a cartouche; solid lines represent passages from one animal to another, dotted lines represent cloning; M = mouse; numbers are the day of infection on which the passage was made; IFA results are shown in brackets.

(a) Percentage prevalence of slender, intermediate and stumpy forms



(b) Total parasitaemia and total numbers of slender, intermediate and stumpy forms

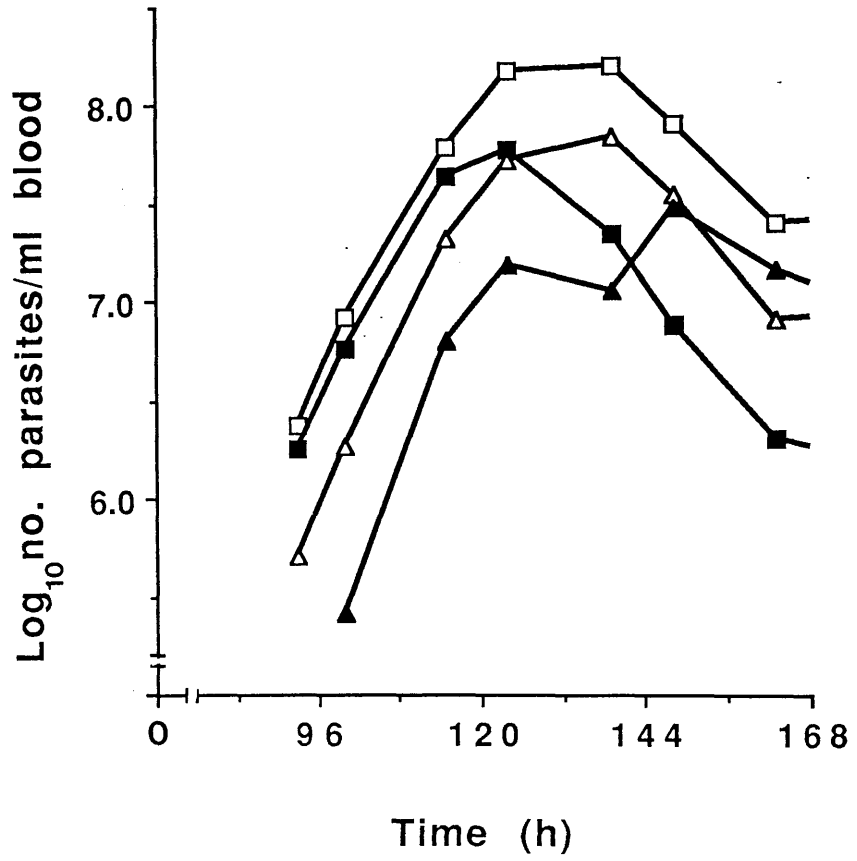


Fig. 2.2. Variations in the relative and absolute abundances of the various morphological types of bloodstream trypanosomes during the first parasitaemic peak after infection of mice with pleomorphic GUTat 7.13. (a) Percentage prevalence of slender, intermediate and stumpy forms (arithmetic means). (b) Total parasitaemia and total numbers of slender, intermediate and stumpy forms ($\log_{10}$ geometric means).

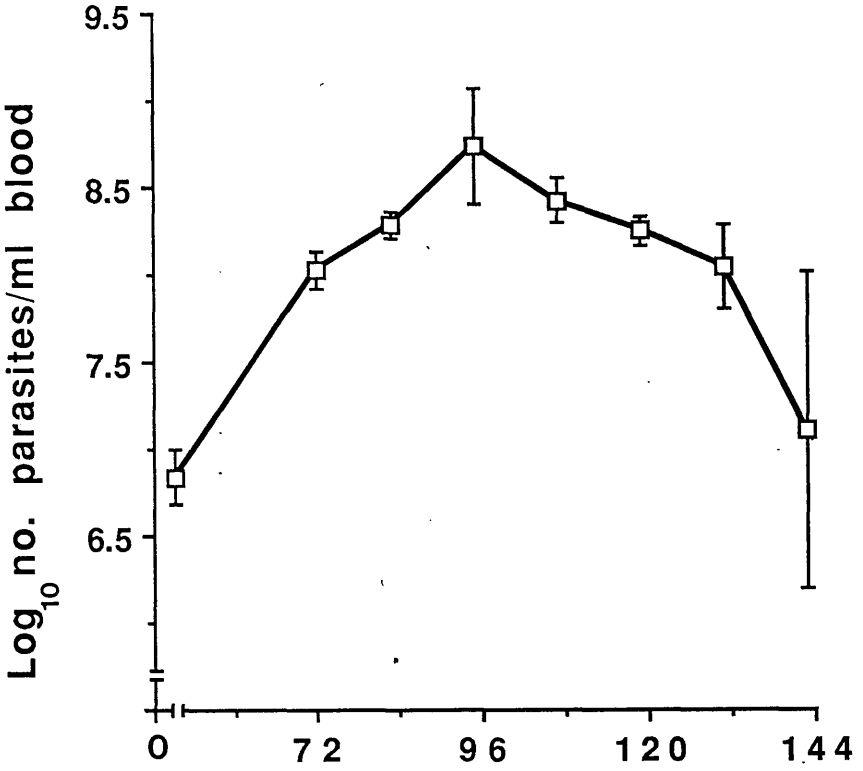
□ total parasitaemia

■ slender forms

△ intermediate forms

▲ stumpy forms

(a) Total parasitaemia against time



(b) Percentage prevalence of slender, intermediate and stumpy forms

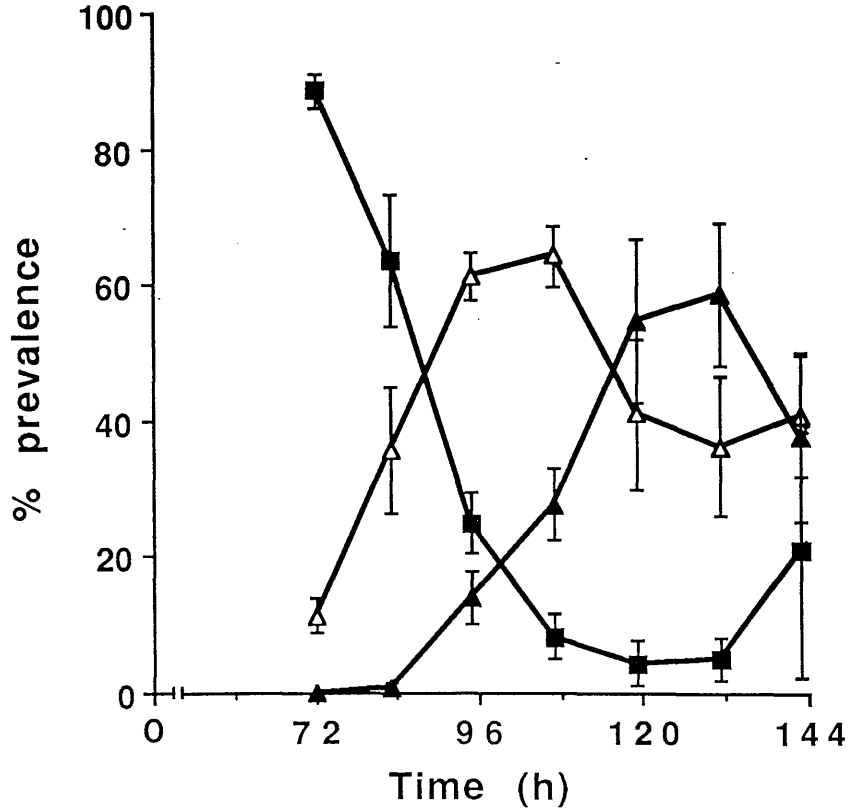


Fig. 2.3. Variations in the absolute and relative abundances of parasites against time in immunocompetent mice infected intraperitoneally with 10^5 pleomorphic GUTat 7.13 parasites. (a) Log_{10} total parasitaemia against time (geometric mean ± 2SE). (b) Percentage prevalence of slender, intermediate and stumpy forms (arithmetic means ± 2SE).

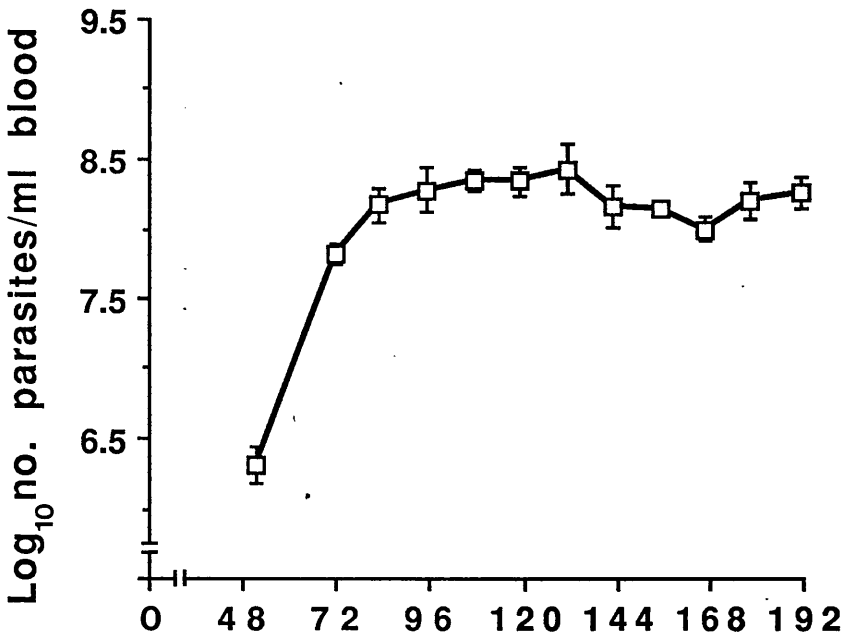
□ total parasitaemia

■ slender forms

△ intermediate forms

▲ stumpy forms

(a) Total parasitaemia against time



(b) Prevalences of morphological forms

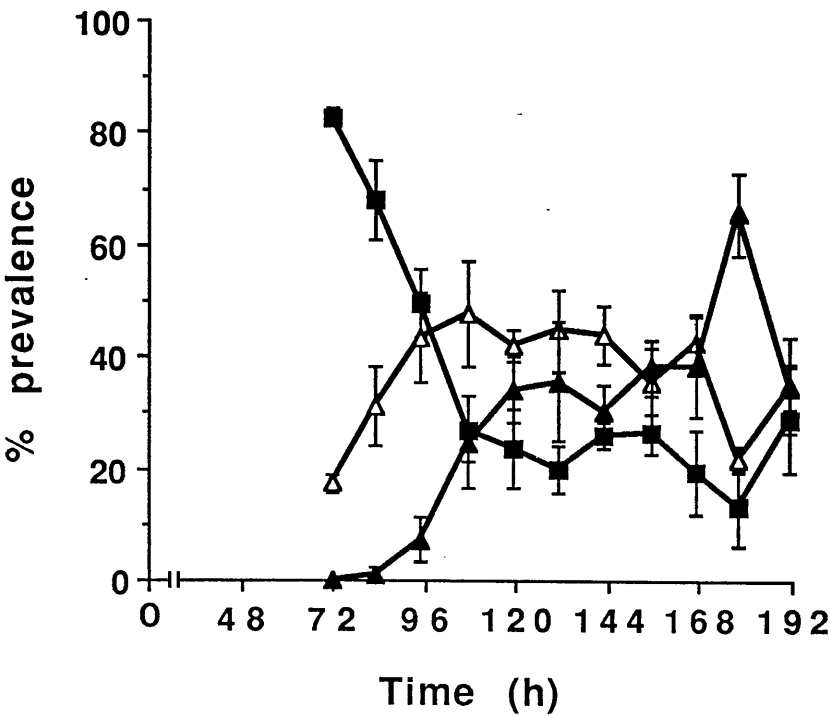


Fig. 2.4. Variations in the absolute and relative abundances of parasites against time in immunosuppressed mice infected intraperitoneally with 10^5 pleomorphic GUTat 7.13 parasites. (a) Log_{10} total number of parasites against time (geometric means ± 2 SE). (b) Percentage prevalences of slender, intermediate and stumpy forms (arithmetic means ± 2SE).

□ total parasitaemia

■ slender forms

△ intermediate forms

▲ stumpy forms

Total parasitaemia against time in immunocompetent and immunosuppressed mice infected with monomorphic ILTat 1.64

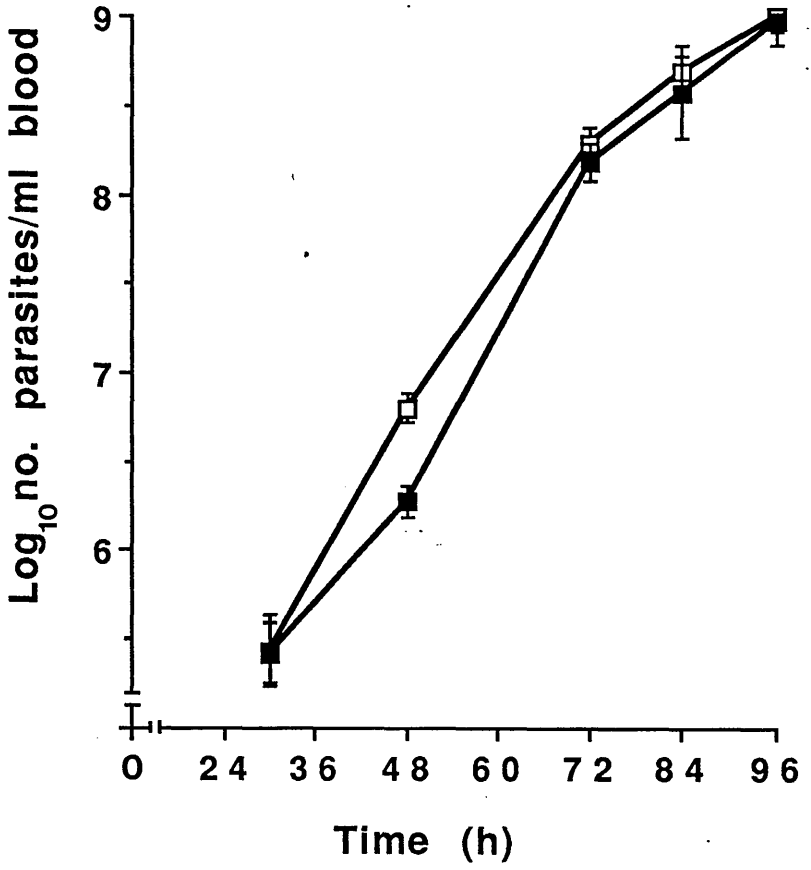


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Fig. 2.5. Log_{10} total number of parasites against time in immunocompetent and immunosuppressed mice infected intravenously with 10^5 monomorphic ILTat 1.64 parasites (geometric means ± 2 SE).

□ immunocompetent mice

■ immunosuppressed mice

Differential localisation of slender and stumpy form trypanosome populations on Percoll gradients formed in situ.

Density profiles for gradients of different Percoll concentrations

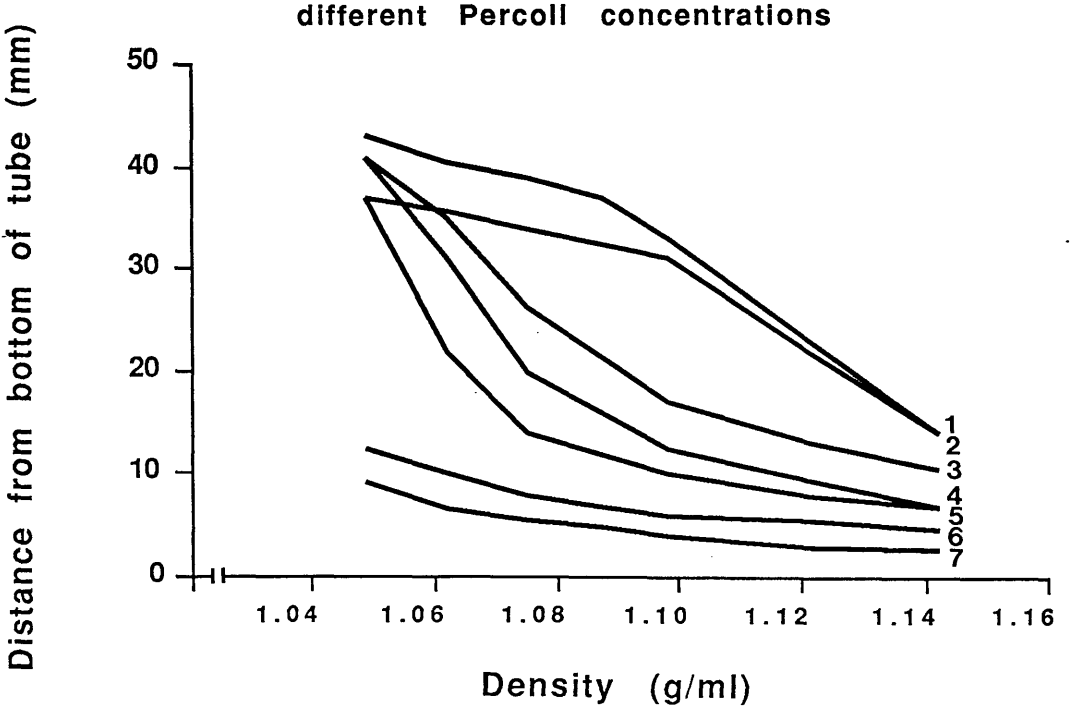

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Fig. 2.6. Differential localisation of slender and stumpy form trypanosome populations on Percoll gradients formed *in situ*. Diagrammatic representations of the two gradients giving best separation of slender and stumpy forms are shown. Meniscus and pellet are the top and bottom of the gradients; the dotted lines show the positions of the density marker beads; and the solid black bars show the positions of the trypanosome-containing bands.

Fig. 2.7. Standardisation experiment for preformed Percoll gradients: density profiles for gradients of different Percoll concentration. Gradients of different average densities containing density marker beads were preformed (23° rotor head, 20 000 x g, 30 mins, 4°C) from mixtures of stock Percoll and 0.15M NaCl. The gradient-containing tubes were numbered 1-7 in order of increasing Percoll concentration. The positions of the density marker beads in each gradient were plotted against their pre-calibrated density values. Tube 5 had the smallest gradient around the approximate density of the trypanosomes.

Centrifugation of predominantly slender and predominantly stumpy populations expressing GUTat 7.13

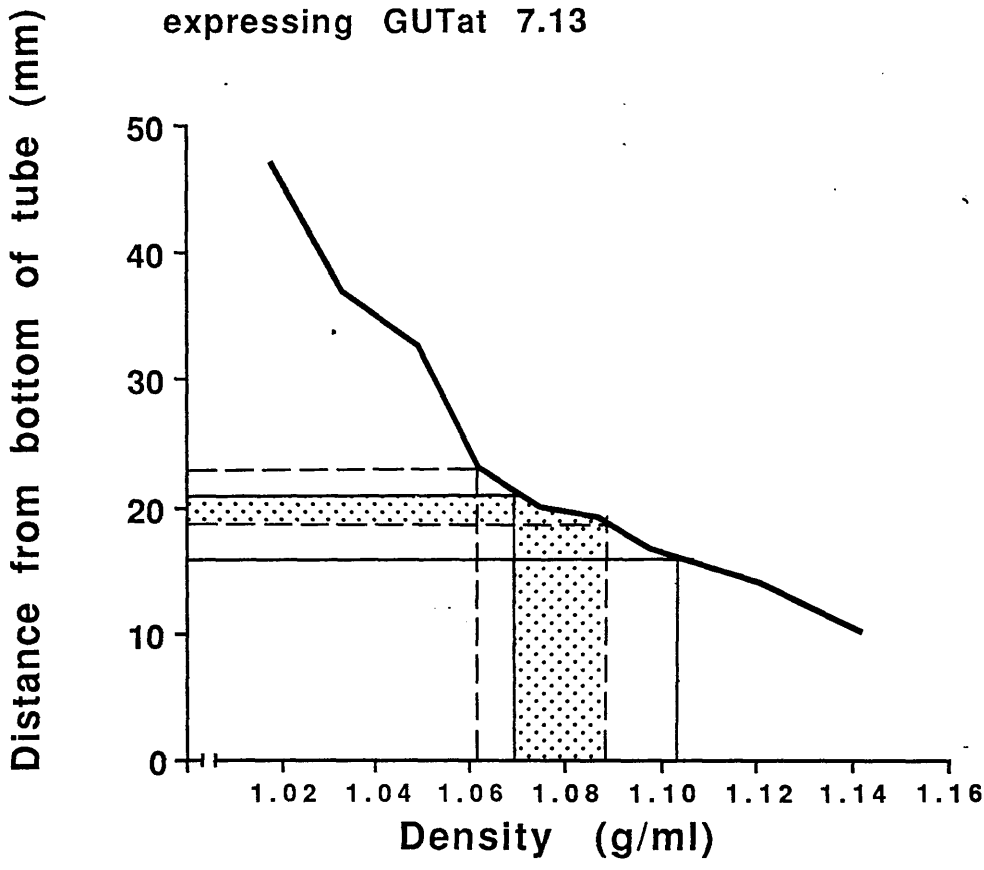

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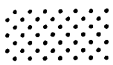
Fig. 2.8. Density profiles of preformed Percoll gradients used for centrifugation of slender and stumpy forms of *T. brucei*.

Predominantly slender (day 4) and predominantly stumpy (day 6) populations expressing GUTat 7.13 were spun through two identical preformed Percoll gradients. A third gradient contained density marker beads. These were used to plot the profile of the density gradient, against which the measured positions of the trypanosome-containing bands were compared.

——— predominantly slender forms

– – – – predominantly stumpy forms

 area of overlap

Chapter 3

EVIDENCE THAT MULTIPLICATION OF BLOODSTREAM *T. BRUCEI* IS NOT LIMITED BY HOST-DERIVED GROWTH FACTOR

3.1 INTRODUCTION

In cyclically transmitted *Trypanosoma brucei* infections of the mammalian host "slender" forms are responsible for growth of the parasitaemia (Lumsden, 1972), and non-dividing "stumpy" forms are infective to the tsetse fly vector (Robertson, 1913b). Parasite lines displaying this morphological variability are termed pleomorphic. Trypanosome strains that have been repeatedly syringe-passaged through laboratory rodents lose the ability to differentiate into stumpy forms and such lines are termed monomorphic. Pleomorphic trypanosome infections in the blood characteristically exhibit repeated cycles of exponential growth followed by a sharp decline in numbers. These fluctuations are caused by the interactions of expanding trypanosome populations undergoing antigenic variation with variable antigen specific immune responses on the part of the host (reviewed by Vickerman, 1985). During each wave of infection slender forms predominate over stumpy forms as the population increases and the converse occurs during remission. This change in relative abundances of the slender and stumpy forms is not caused by an increase in the total population of stumpy forms, but is due to a reduction of the absolute abundance of slender parasites (Balber, 1972; see section 2.2).

Black and colleagues obtained evidence that led them to postulate that the change in predominance from slender to stumpy forms was due to an increased rate of differentiation in the parasite population (Black *et al.*, 1983). They proposed that the division of slender forms was

maintained by a high avidity interaction between slender forms and a host-derived growth factor (Black, Jack & Morrison, 1983), and that differentiation resulted from the depletion of this factor by the expanding population (Black *et al.*, 1985). The functional role of such a growth factor is of interest because manipulation of it could control the level of infection, which in turn correlates with the extent of damage to the host.

The object of the experiments described in this chapter was to test directly the hypothesis that the depletion of a growth factor prevents slender form replication. This was achieved by superimposing a challenge dose of slender trypanosomes onto a preexisting infection in which most of the parasites were stumpy. The predominance of stumpy forms over slender forms in the primary infection indicates, according to the hypothesis, that the putative growth factor has been depleted. If there is a requirement for this growth factor to maintain division of slender forms, then the challenge trypanosomes should not grow.

The experiments were facilitated by the use of trypanosome lines that have been manipulated such that they possess an unusual combination of properties: they are antigenically homogeneous and pleomorphic. Since the primary and challenge populations each homogeneously express a different VAT the fate of each in doubly-infected mice can be monitored.

3.2 MATERIALS AND METHODS

3.2.1 Trypanosomes

Five cloned lines, each homogeneously expressing a single VAT, were used in these experiments. The pleomorphic lines GUTats 7.2 and 7.13 and the monomorphic line GUTat 7.2 were all described in Chapter 2. Two other parasite lines were also used: a monomorphic line expressing

GUTat 7.1, derived from stock EATRO 2340 (Barry, Crowe & Vickerman, 1985); and a pleomorphic *T. brucei* line AnTat (Antwerp *Trypanozoon* antigen type) 1.8, derived from stock EATRO 1125 (Le Ray *et al.*, 1977).

3.2.2 Monoclonal antibodies and antisera

Trypanosome VATs were identified using previously described monoclonal antibodies (Turner, Barry and Vickerman, 1986) in immunofluorescence reactions. The monoclonal antibodies used were Glasgow University Protozoology Monoclonal antibodies (GUPMs) 17.1, 18.7 and 27.1 which are specific for GUTats 7.13, 7.2 and 7.1, respectively.

A specific rabbit antiserum was raised against AnTat 1.8 using a standard procedure (Van Meirvenne, Janssens and Magnus, 1975).

3.2.3 Immunofluorescence reactions

The antigenic identity and homogeneity of the parasite populations used in the following experiments were confirmed by indirect IFT (Turner, Barry & Vickerman, 1988, based on the method of Van Meirvenne, Janssens & Magnus, 1975). Where parasites in whole blood were used, thin smears were stained. Where trypanosomes had been isolated from the blood, smears were made onto glass slides which had been coated with poly-L-lysine to give a proteinaceous surface to which trypanosomes could attach. In both cases the smears were air-dried and fixed in acetone for 15 min. Either the rabbit antisera described above (at 1/100 dilution) or the undiluted supernatant of monoclonal antibody producing mouse cell culture was applied to the slides as the first antibody. FITC-conjugated sheep anti-mouse immunoglobulin (Scottish Antibody Production Unit) at 1/50 dilution in PBS containing propidium iodide (1/10000) was used as the second antibody.

3.2.4 Experimental design

Each experiment consisted of three groups of three to six Balb/c female mice. On day 0 groups 1 and 3 were inoculated with approximately 10^5 pleomorphic trypanosomes i.p. while group 2 mice remained uninfected. On day 5 of the infection when stumpy forms predominated over slender forms, groups 1 and 2 were inoculated i.v. with 2 x 10^7 trypanosomes of a different line and expressing a different VAT. Table 3.1 shows the combination of trypanosome lines used in each of four experiments. In three experiments the challenge infections were of monomorphic lines and in one experiment the challenge was slender form trypanosomes from a pleomorphic line. Thus, groups 2 and 3 served as controls showing the time-course of parasitaemia of each of the primary and challenge infections and with which the experimental results of group 1 could be compared. The fate of each population in the double infection of group 1 could be determined serologically because the primary and challenge inocula were both antigenically homogeneous populations expressing different VATs.

Blood smears were taken from mice for immunofluorescence analysis, and accurate counts of the population density were made using an improved Neubauer haemocytometer and 0.85% ammonium chloride as diluent. These two procedures were carried out daily during the first 110-120 hours of the experiments, and thereafter approximately 12 hourly until the end of the experiments.

A second set of blood smears taken at each time-point were fixed in methanol for 2 minutes and Giemsa-stained. These were used to determine the relative abundances of slender and stumpy forms present in the trypanosome populations using the differential morphological

criteria of Wijers (1959a,b).

3.2.5 Statistical analysis

The population densities at each time-point were expressed as the geometric means ± 2 SE for each experimental group. The percentage abundance of a VAT within each group was based on counts of approximately 200 trypanosomes per mouse. These abundances were expressed as means ± 2 SE. The absolute number of parasites of the challenge VAT present in a mixed infection was calculated:
(proportion of the population expressing that VAT x population density)
The population doubling times (PDT) of the monomorphic parasite populations were calculated by determining the rate of replication, b, by least squares exponential regression analysis, and from the equation $PDT=\log_e 2/b$.

In experiments 1-3 the growth rates of monomorphic parasites alone (group 2) or when superimposed onto a pleomorphic infection (group 1) were compared by analysis of covariance.

The percentage abundance of slender and stumpy forms in populations were based on counts of approximately 100 trypanosomes per mouse and were expressed as means ± 2 SE.

3.3 RESULTS

The hypothesis being tested (Black *et al.*, 1985) proposes that stumpy forms predominate just after the peak of infection because a growth factor required for slender form division has been depleted. In the experiments described here the experimental groups were challenged with slender form trypanosomes at a point in the infection when non-dividing stumpy forms predominated, i.e. when the growth factor had

theoretically been depleted. Table 3.2 shows the proportions of slender and stumpy forms present in the experimental group of each experiment immediately before the challenge doses were administered. Wijers (1959a,b) categorised trypanosomes as dividing, slender, intermediate or stumpy. Table 3.2 shows dividing and slender forms under the heading "slender" and does not show the proportion of intermediate forms. During the exponential growth phase of a parasitaemic wave over 95% of trypanosomes in the population were slender forms, therefore Table 3.2 shows a dramatic decrease in the proportion of slender forms on day 5 of infection even in experiment 3 where stumpy forms were only approximately 40% of the population at the time of challenge.

3.3.1 Superimposition of monomorphic GUTat 7.2 onto a preexisting infection with AnTat 1.8

The results of experiment 1 are shown in Fig. 3.1. The mean total parasitaemia in each group was plotted against time after infection (Fig. 3.1a). Mice in Group 1 received a primary inoculum of pleomorphic AnTat 1.8 at t=0 h and a challenge inoculum of monomorphic GUTat 7.2 at t=125 h. The parasitaemia increased to peak value on days 4-5, decreased slightly on day 6 and then increased again. In the control group which was given only a primary infection (group 3), however, the mean parasitaemia decreased steadily after day 5. In the other control group which received only a challenge infection (group 2) the mean parasitaemia increased exponentially, indicating that the rise in parasitaemia on days 6-8 of infection in the experimental group was likely to have been caused by the growth of GUTat 7.2 trypanosomes in the challenge inoculum.

The results of immunofluorescence analysis of group 1 with antibodies

* The reason for detection of only 34% of trypanosomes at t=154 h in experiment 1 is unknown.

specific for GUTat 7.2 and AnTat 1.8 are shown in Fig. 3.1b. No GUTat 7.2 specific fluorescence was detected before t=125 h, while after this time-point the proportion of trypanosomes expressing GUTat 7.2 increased rapidly as the percentage of those expressing AnTat 1.8 decreased. These results show that the increase of parasitaemia in group 1 was indeed caused by growth of the challenge GUTat 7.2 population.

Fig. 3.1c shows the growth rates of the challenge parasites in groups 2 and 1, i.e. alone and superimposed upon an existing infection. The growth rate (slope) of the monomorphic challenge in group 1 was significantly slower than in group 2 (Table 3.3).

Experiment 3 was a replicate of experiment 1 i.e. a monomorphic challenge population of trypanosomes expressing GUTat 7.2 was superimposed onto a primary infection of pleomorphic AnTat 1.8 trypanosomes. The results of this experiment were similar to those of experiment 1 except that analysis of covariance revealed no significant difference between the growth rates of the challenge GUTat 7.2 trypanosome populations in the doubly- and singly-infected groups.

3.3.2 Superimposition of monomorphic GUTat 7.1 onto a preexisting infection with GUTat 7.13

The experiment described above was repeated to confirm that the result was not VAT or stock specific (Table 3.1). The results of experiment 2 are shown in Fig. 3.2. Groups 1 and 3 received a primary inoculation of pleomorphic GUTat 7.13 i.p. at t=0 h: groups 1 and 2 were inoculated i.v. with the challenge trypanosome line, monomorphic GUTat 7.1, at t=116 h. Fig. 3.2a shows mean total parasitaemia of each group against time. These results are very similar to those shown in Fig. 3.1a except that the second phase of exponential growth in the

doubly-infected group was delayed by approximately 20 h compared with the challenge control group.

Fig. 3.2b shows results of immunofluorescence analysis of group 1. As the infection progressed from t=0 to t=116 h the proportion of parasites expressing GUTat 7.13 decreased slightly. This was probably caused by some of the parasites switching to expression of unlabelled antigen types. However, no GUTat 7.1 was expressed until after t=116 h when parasites expressing this VAT were inoculated into the mice. After that time-point the proportion of parasites expressing GUTat 7.1 increased rapidly reaching 99% at t=187 h and there was a concomitant rapid decline in the relative abundance of GUTat 7.13.

Fig. 3.2c shows the growth rates of GUTat 7.1 in groups 2 and 1: i.e. alone and when superimposed upon an existing infection. These results indicate that there was little difference in the growth rates of the challenge GUTat 7.1 trypanosomes between the doubly- and singly-infected groups. Covariance analysis showed this difference to be not statistically significant (Table 3.3).

3.3.3 Superimposition of pleomorphic GUTat 7.13 onto a preexisting infection with GUTat 7.2

In experiment 4 the primary infection was pleomorphic GUTat 7.2 and the challenge inoculum was pleomorphic GUTat 7.13. The challenge infection was given at t=120 h of the primary infection, at which point the parasitaemia had already passed its peak value and was declining. Immunofluorescence testing of blood smears taken from the doubly-infected group at time intervals after the challenge infection had been given showed an increasing level of GUTat 7.13 (the challenge VAT), although there was wide variation between individual mice in the group: by t=168 h none of the animals was expressing the primary

*

A fifth experiment (Table 3.4) also used a pleomorphic line as the challenge. In that experiment pleomorphic GUTat 7.2 was superimposed onto a preexisting infection of a pleomorphic line AnTat 1.8. As Table 3.4 shows, the level of parasitaemia in the doubly-infected experimental group after the challenge (at t=144 h and t=168 h) reached a plateau that was lower than the parasitaemia in the challenge control group, but was dramatically higher than the parasitaemia in the primary infection control group. The growth observed in the doubly-infected group correlated with the higher proportions of slender form trypanosomes in this group compared to the primary challenge control group, and immunofluorescence analysis confirmed that the growth was due to proliferation of trypanosomes bearing the challenge VAT.

infection VAT, GUTat 7.2, but the four animals expressed 38, 78, 62 and 61% GUTat 7.13. Strikingly, even where percentage expression of the challenge VAT was high, so also was the prevalence of stumpy forms, i.e. the challenge trypanosomes were transformed into stumpy forms soon after being injected into the already infected animals. In contrast, the challenge VAT control group was over 80% GUTat 7.13 at t=168 h and comprised mainly slender forms. The primary infection control group did not contain any GUTat 7.13 at any time, and the proportion of the primary infection VAT, GUTat 7.2, dropped to less than 10% by t=192 h. The large variation in results of individual mice in the experimental group precluded statistical analysis of the rates of growth of trypanosomes of the challenge inocula in singly-and doubly-infected mice, therefore this experiment is not represented on Table 3.3.

3.4 DISCUSSION

From these experiments it was concluded that cessation of division and differentiation to stumpy forms are not necessarily consequences of depletion of a host-derived growth factor.

During the course of a single parasitaemic wave stumpy forms come to predominate over slender forms. This predominance is caused by a decrease in absolute abundance of slender forms. Black *et al.* (1985) postulated that this decrease is caused by a cessation in slender form multiplication, which itself is due to depletion of an as yet undefined host serum factor. If the decrease in slender form parasites observed after peak parasitaemia was due to depletion of a substance required for division, then the parasite challenges given to the group 1 (doubly-infected) animals in these experiments would not have become established infections. In all the experiments described here,

however, trypanosomes of the challenge inoculum did establish infections.

The combinations of VATs used in experiments 1-4 demonstrated that the effect observed was neither stock- nor VAT-specific. The trypanosome line, AnTat 1.8, used for the primary infections in experiments 1 and 3 is from stock EATRO 1125, whereas GUTats 7.13 and 7.2, the primary infection lines used in experiments 2 and 4, are from the serologically unrelated stock EATRO 2340. All three of the challenge infection lines, GUTats 7.1, 7.2 and 7.13, are from stock EATRO 2340. That is, in experiments 1 and 3 the primary and challenge VATs are from different stocks, while in experiments 2 and 4 the primary and challenge VATs are from the same stock. In all four experiments the challenge VATs grew, therefore the effect is not stock-specific. The use of challenge infections expressing three different VATs, GUTats 7.1, 7.2 and 7.13, shows that the ability of trypanosomes to grow when superimposed upon a preexisting infection is not limited to one VAT.

There is evidence that the growth rate of a challenge infection may be slower in superimposed trypanosome infections (Seed, 1978) and that onset of detectable parasitaemia may be delayed. Trypanosomes of the challenge inocula in the experiments described here had slower growth rates, but the difference was statistically significant only in experiment 1 (see Table 3.3). A delay in the onset of growth of the challenge infection was detected in only one experiment (experiment 2) where there was a time lag of approximately 20 h (see Fig. 3.2a). These results suggest that reduced growth rate and delayed onset of growth are not manifestations of the same phenomenon.

To exclude the possibility that monomorphic trypanosomes may differ from pleomorphic parasites in their response to the putative growth

factor, a challenge inoculum of slender form pleomorphic trypanosomes was given in experiment 4. In this experiment patent infections of trypanosomes of the challenge VAT were detected, indicating that growth had occurred. This provides evidence that monomorphic and pleomorphic parasites do not differ qualitatively. Supporting evidence that slender forms from pleomorphic lines resemble parasites from monomorphic lines comes from the observation of Black *et al.* (1983) that a line of parasites that was monomorphic in rodents readily produced stumpy forms in cattle. The ultrastructure and energy metabolism of monomorphic parasites and slender forms are also similar (reviewed in Vickerman, 1985).

Although growth of the challenge VAT was observed in experiment 4 in the doubly-infected mice, the level of parasitaemia achieved was less than in the singly-infected control mice and the trypanosomes were mainly of stumpy morphology. These aspects of the results may be constituted as providing some support for the hypothesis of Black and colleagues. If that hypothesis is indeed correct then the results presented here show that depletion of the putative growth factor must be a very slight and/or transitory event. The results presented here suggest that depletion of a growth factor is unlikely to cause the observed decrease in absolute numbers of slender forms during a parasitaemic wave. There are two other possible explanations for this decrease: an increased rate of differentiation to stumpy forms, and/or increased death rate of slender forms. The former explanation is unlikely because the rate of production of stumpy forms is greatest during the rising phase of a parasitaemia, at the same time as exponential growth of slender forms occurs (Balber, 1972; Chapter 2.2). Evidence for the latter explanation is described and discussed in Chapter 4.

The results presented in this chapter should not be misinterpreted as implying that trypanosome division is insensitive to the effects of growth factors. There is, indeed, clear evidence that trypanosomes do respond to exogenous changes (Hide *et al.*, 1989; Coppens *et al.*, 1987; Black & Vandeweerd, 1989). The evidence put forward here indicates only that depletion of a putative growth factor does not inhibit slender form trypanosome multiplication in the mammalian bloodstream.

A growth factor such as Black and colleagues postulate would have considerable significance for alleviation of trypanosome-induced pathogenesis. The level of morbidity suffered by the mammalian host is directly related to the parasite burden. Manipulation of a host-derived growth factor to inhibit trypanosome replication and therefore reduce parasitaemia would be an important therapeutic breakthrough. Work has been carried out to investigate the relationship of trypanosomes with host growth factors. Hide *et al.* (1989) have shown that EGF binds to both procyclic and bloodstream trypanosomes. Coppens *et al.* (1987) investigated uptake of host serum proteins by *T. brucei*. They found that total plasma proteins and serum albumin are taken up by fluid endocytosis while LDL and transferrin are taken up by a calcium-dependent, receptor-mediated process which occurs only in the flagellar pocket. Trypanosomes may require large amounts of cholesterol to support their rapid multiplication, and the receptor-mediated uptake of LDL may be important in boosting sterol levels. However, this is not evidence that LDL is a growth factor (as distinct from an essential nutrient) for bloodstream trypanosomes, only that growth is limited in absence of LDL.

One pathogenic effect of bloodstream trypanosomes is that they aggregate and lyse platelets, causing severe thrombocytopenia at peak parasitaemia (Davis, 1982). This suggested a role for PDGF in control

of trypanosome growth and/or differentiation. Davis and colleagues suggested that PDGF may negatively regulate trypanosome growth: lysis of platelets at high parasitaemia releases large amounts of PDGF which may then inhibit trypanosome replication or promote differentiation. (This is discussed in section 1.6.4.) It remains to be seen whether EGF and/or PDGF are specific ligands important in regulation of trypanosome multiplication.

It is conceivable that trypanosome growth may be controlled partly or wholly by the feedback of substances produced by the parasites themselves rather than by the host. The results presented in this chapter preclude the possibility that there is any substance produced within populations which prevents division of slender forms or promotes differentiation. However, the results do not rule out the possibility that the parasites produce substances which promote division of slender forms, only that depletion of such substances does not result in differentiation.

Table 3.1. Trypanosome populations used in each experiment.
In each experiment a pleomorphic infection was initiated by i.p. inoculation of 10^5 parasites. On day 5 (when division of slender forms had virtually ceased) a challenge dose of $2x10^7$ i.v. trypanosomes was superimposed on the first infection.

Exp.	Trypanosome variable antigen type	
	pleomorphic primary infection	monomorphic challenge
1	AnTat 1.8	GUTat 7.2
2	GUTat 7.13	GUTat 7.1
3	AnTat 1.8	GUTat 7.2
		pleomorphic challenge
4	GUTat 7.2	GUTat 7.13

Table 3.2. Morphology of trypanosome populations in primary infections at the time of inoculation of challenge infections (mean ± 2 standard errors).

Experiment	n	morphology of population (%)	
		slender forms	stumpy forms
1	5	14.8 ± 7.1	58.2 ± 3.5
2	6	29.1 ± 6.5	56.8 ± 4.4
3	4	40.8 ± 13.4	39.5 ± 5.2
4	3	21.5 ± 3.0	66.3 ± 7.7

Table 3.3. Population doubling times of monomorphic challenge infections in double and single (control) infections.
The population doubling times (PDT) of the monomorphic challenge infections were calculated as described in the text. Analysis of covariance was used to determine whether the differences in the growth rates of monomorphic populations between singly- and doubly-infected animals were significant.

Experiment	Trypanosome VAT		PDT(h)	F	p
	Primary	Challenge			
1	1.8	7.2	8.31	19.87	<0.001
	-	7.2	7.04		
2	7.13	7.1	9.60	1.24	>0.05
	-	7.1	7.55		
3	1.8	7.2	7.33	0.72	>0.05
	-	7.2	6.82		

Table 3.4. Superimposition of a pleomorphic line GUTat 7.2 onto a preexisting infection of pleomorphic AnTat 1.8.
Experimental groups were as described for experiments 1-4.
t=0 h: 10^5 pleomorphic AnTat 1.8 trypanosomes i.p. to each mouse in groups 1 and 3.
t=120 h: 2 x 10^7 pleomorphic GUTat 7.2 trypanosomes i.v. to each mouse in group 2.

Doubly-infected experimental group (group 1, n=5):

Time (h)	$\log_{10}$ parasitaemia	% slender forms	% stumpy forms	%expressing challenge VAT
96	8.0±0.4	-	-	-
120	8.5±0.2	53.0± 2.7	47.0± 2.7	18.5± 9.6
144	7.9±0.2	40.2±15.8	59.8±15.8	65.4±32.2
168	8.0±0.3	67.1±10.4	32.9±10.4	98.4± 1.4

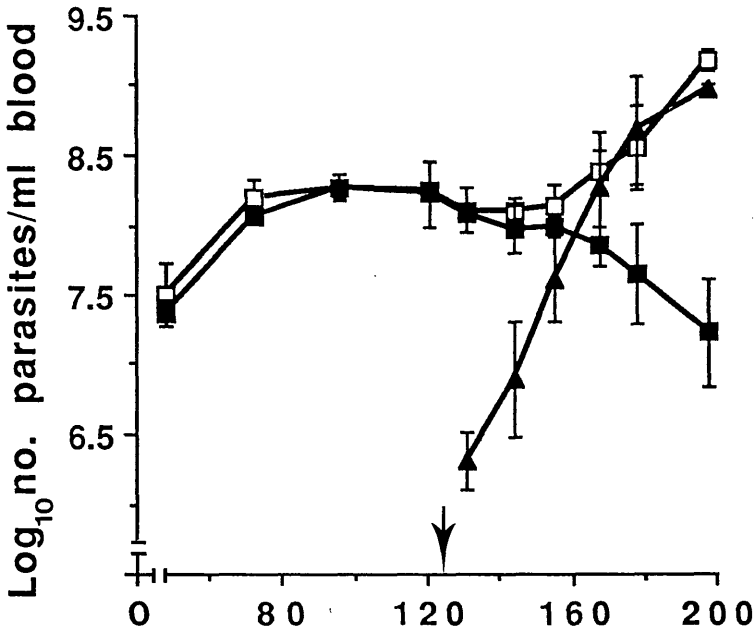
Challenge VAT control group (group 2, n=5):

Time (h)	$\log_{10}$ parasitaemia	%expressing challenge VAT
120	7.4±0.3	94.4±11.2
144	8.7±0	97.6± 2.8
168	8.7±0	99.0± 0.6

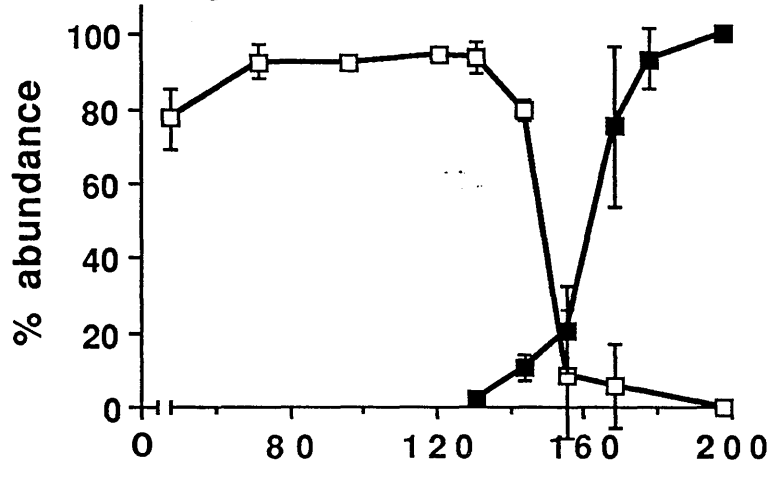
Primary infection control group (group 3, n=5):

Time (h)	$\log_{10}$ parasitaemia	% slender forms	% stumpy forms	%expressing challenge VAT
96	8.1±0.2	-	-	-
120	8.2±0.2	38.5± 6.4	61.5± 6.4	0
144	(n=2; i.e. no trypanosomes present in 3 mice):			
	6.2±1.1	12.0±10.0	88.0±10.0	0
169	no trypanosomes present			

(a) Parasitaemia against time



(b) Percentage abundance of primary and challenge VATs



(c) Growth rates of monomorphic parasites

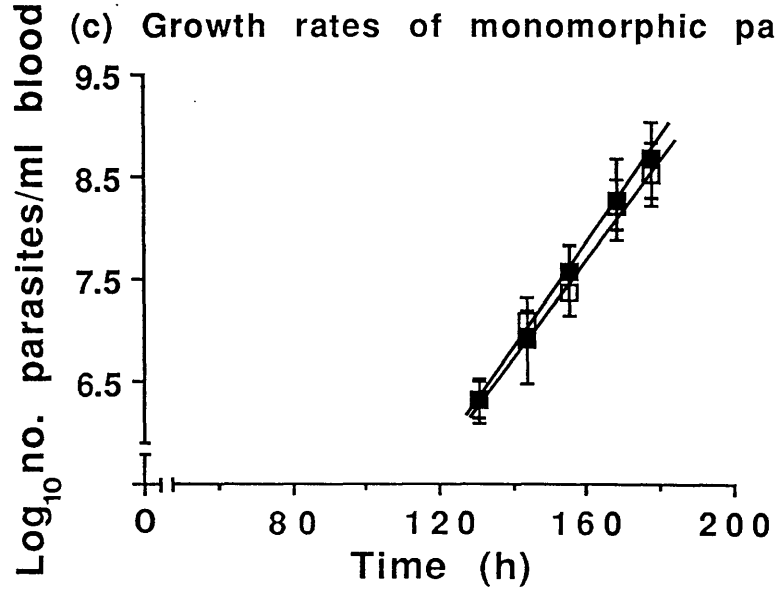


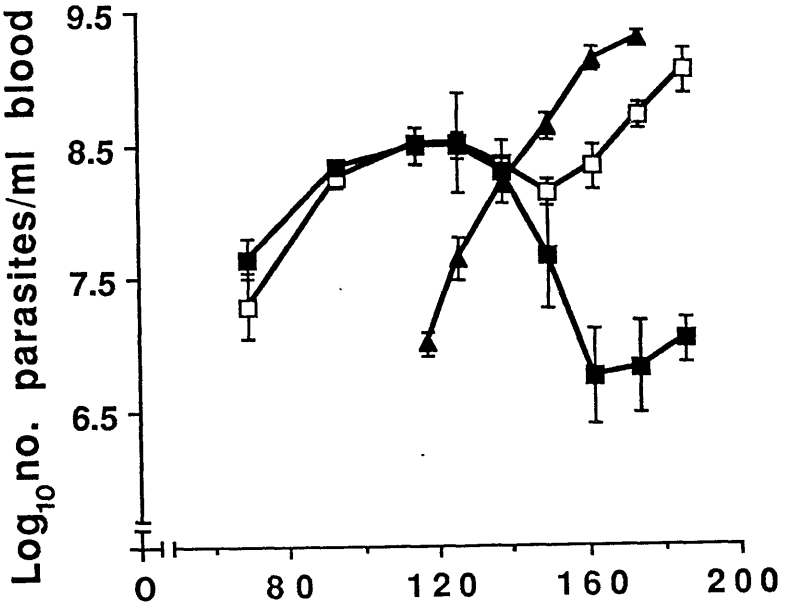
Fig. 3.1. Superimposition of a monomorphic GUTat 7.2 infection onto an existing pleomorphic infection with AnTat 1.8 in mice. In groups 1 and 3, mice were infected with pleomorphic AnTat 1.8 trypanosomes at t=0 h. Mice in group 2 were not infected. At t=125 h (arrow) the mice in groups 1 and 2 were injected intravenously with monomorphic GUTat 7.2 parasites. (a) Geometric mean ± 2 SE $\log_{10}$ number of parasites/mouse in each group against time (n=5). (b) Mean ± 2 SE percentage of total number of parasites/mouse in group 1 expressing AnTat 1.8 (□) or GUTat 7.2 (■) against time. (c) Linear regression of numbers of monomorphic GUTat 7.2 parasites/mouse against time in groups 1 (□) and 2 (■) from t=125 h onwards.

□ doubly-infected experimental group (group 1)

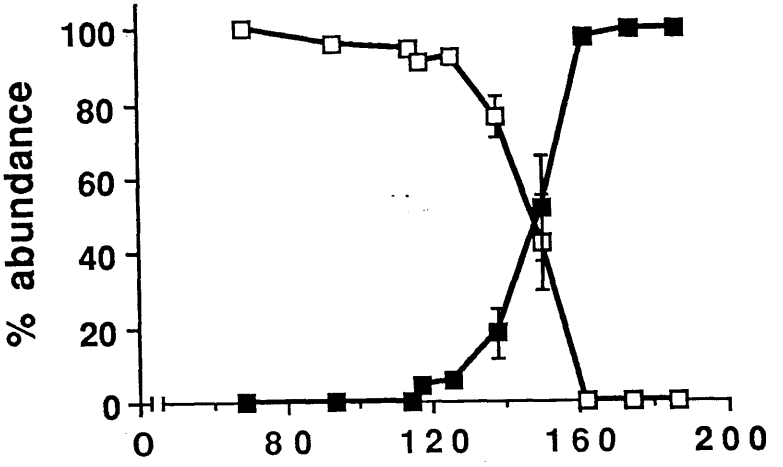
▲ monomorphic control group (group 2)

■ pleomorphic control group (group 3)

(a) Parasitaemia against time



(b) Percentage abundance of primary and challenge VATs



(c) Growth rates of monomorphic parasites

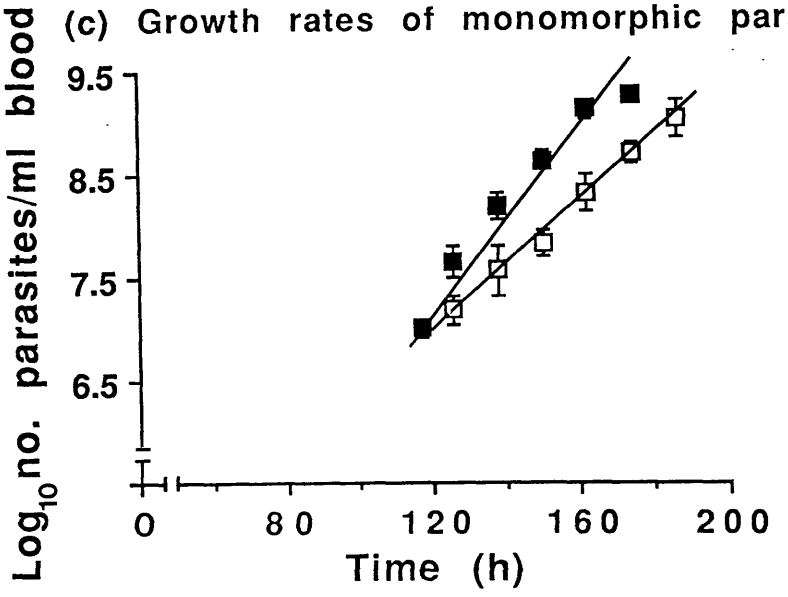


Fig. 3.2. Superimposition of a monomorphic GUTat 7.1 infection onto an existing pleomorphic GUTat 7.13 infection in mice. In groups 1 and 3 mice were infected with pleomorphic GUTat 7.13 parasites at t=0 h. Mice in group 2 were not infected. At t=116 h monomorphic GUTat 7.1 parasites were injected intravenously into each animal in groups 1 and 2. (a) Mean ± 2 SE $\log_{10}$ number of parasites/mouse in each group against time. (b) Mean ± 2 SE percentage of parasites/mouse in group 1 expressing GUTat 7.1 (■) or GUTat 7.13 (□) against time. (c) Linear regression of numbers of monomorphic GUTat 7.1 parasites/mouse against time in groups 1 (□) and 2 (■) from t=116 h onwards.

□ doubly-infected experimental group (group 1)

▲ monomorphic control group (group 2)

■ pleomorphic control group (group 3)

Chapter 4

IMMUNE MECHANISMS INVOLVED IN KILLING OF *T. BRUCEI IN VITRO*

4.1 INTRODUCTION

Results presented and discussed in Chapter 3 showed that the change from predominance of slender forms to predominance of stumpy forms at the parasitaemic peak is unlikely to be due to depletion of a host-derived growth factor. An alternative possibility, that the change is due to selective killing of slender forms, is investigated in this chapter.

There is some slight evidence that stumpy forms are more resistant to immune mediated killing than slender forms. Balber (1972), in his discussion of data obtained from monitoring the morphological profiles of parasite populations in mice, noted that immunosuppressive agents increase the numbers of slender and dividing forms, and suggested that these forms are not subject to attack by specific antibody in immunosuppressed animals. Also, preliminary observations have indicated that slender form parasites are lysed more quickly than stumpy form parasites *in vitro* by specific antibody plus complement (Barry & Vickerman, 1977), and by the human serum factor responsible for resistance to *Trypanosoma brucei brucei* (Vickerman & Barry, 1982). These observations of differential lysis may be particularly significant because antibody-dependent complement-mediated lysis is probably a major anti-trypanosome effector mechanism: very rapid lysis of challenge parasites has been observed in drops of tail-blood taken from mice previously immunised with homologous parasites (Vickerman & Barry, 1982). The second major immune effector mechanism is phagocytosis of opsonised trypanosomes by macrophages. This has been observed both *in vitro* (Lumsden & Herbert, 1967; Mosser & Roberts,

1982), and *in vivo* (MacAskill *et al.* 1980).

To determine whether immune effector mechanisms might be responsible for selective killing of slender forms, two sets of experiments were conducted *in vitro*. The two assay systems used each correspond to one of the effector mechanisms described above that are considered to be of major importance *in vivo*. The very preliminary finding of Barry & Vickerman (1977) described above was confirmed *in vitro* by exposing trypanosomes from day 3 and day 6 post-infection parasitaemias to specific antibody in the presence of complement. The results obtained were then extended and quantified by incubating predominantly slender, predominantly stumpy, and morphologically mixed populations of bloodstream *T. brucei* with specific antibody and complement in standardised conditions, and observing how quickly parasites were killed. Experiments were also conducted to investigate whether there were any quantitative differences in the rates of phagocytic clearance of slender and stumpy forms. An assay for attachment *in vitro* was devised using macrophages derived from murine peritoneal exudates. Slender or stumpy form parasites were incubated with the macrophages in the presence of specific antibody with or without exogenous complement.

4.2 MATERIALS AND METHODS

4.2.1 Trypanosomes and VAT-specific antibodies

The two pleomorphic parasite lines used in these experiments each express one dominant VAT, either GUTat 7.2 or GUTat 7.13, during the first peak of infection in a mammalian host. The derivations of these lines were described in Chapter 2. Rabbit antisera raised against these parasites using the standard procedure of Van Meirvenne, Janssens & Magnus (1975) were used in the lysis and opsonisation

experiments, as was the GUTat 7.2-specific mouse monoclonal antibody GUPM 18.7 (Turner, Barry & Vickerman, 1986). The optimum dilutions for the use of each antiserum or ascitic fluid were established in preliminary experiments using serial dilution titration with constant numbers of homologous slender form parasites (results not shown). The highest dilution giving greater than 90% lysis was used in the following experiments. Where required, antisera were heat-inactivated (to destroy complement components) by incubation at 56°C for 30 mins.

4.2.2 Complement-mediated lysis

Blood containing slender form parasites was obtained from female CFLP mice on the third day after infection with GUTat 7.2 and stumpy forms were obtained on day 6 post-infection. For the lysis experiments, blood containing either slender or stumpy form parasites was mixed with fresh guinea pig serum (as a source of complement) either in the absence of antibody or with GUPM 18.7 ascitic fluid at a final dilution of 1/500. On days 3 and 6 of infection there were approximately 2.5×10^8 parasites/ml of blood, therefore 5 μl of blood in a total final sample volume of 50 μl gave an approximate parasite density of 2.5×10^7/ml. Single-drop samples were taken at 10 or 15 min. intervals and the proportion of lysed parasites was counted. Dead parasites are easily distinguishable as pale, still "ghosts" among the highly motile living parasites. Negative control experiments were conducted in parallel in which trypanosomes (slender forms only) expressing a heterologous antigen type (GUTat 7.13) were used.

The reciprocal experiment, using GUTat 7.13 and its homologous lytic rabbit antiserum (diluted 1/100 in fresh guinea pig serum) with GUTat 7.2 parasites as the heterologous negative control was also conducted.

A more sophisticated version of the experiment described above was

also carried out. Morphologically mixed populations were created by mixing the day 3 blood containing slender forms with the day 6 blood containing stumpy form parasites in various ratios (3:1, 1:1 and 1:3). The exact morphological compositions of the day 3 and day 6 blood samples and of the mixtures were determined by differential counting of Giemsa-stained smears (based on the method of Wijers, 1959). Thus, five experimental populations were tested for GUTat 7.2. Parasites at an approximate final density of 10^7/ml were incubated at room temperature in the presence of monoclonal ascitic fluid GUPM 18.7 diluted 1/500 in fresh guinea pig serum. Samples were examined microscopically at approximately 10 min. intervals and the proportions of dead parasites were enumerated as before.

To control for the possibility that there was some difference between parasites on day 3 of infection and parasites on day 6 other than their morphology, the lysis experiments were repeated using stumpy form parasites taken from the bloodstream of a mouse on day 6 after infection with GUTat 7.2 and slender form parasites taken from the lymph nodes of the same mouse also on day 6. This follows from the observation that extravascular sequestration of slender forms occurs (Walker, 1964; Balber, 1972). Trypanosomes from the blood and lymph nodes of mice infected with GUTat 7.13 were used as negative controls. Parasites were isolated from the blood using the DEAE ion-exchange purification method of Lanham & Godfrey (1970), washed and resuspended in PSG, the principal energy source for bloodstream parasites. Cervical, inguinal and mesenteric lymph nodes were removed from the animals and crushed in a Dounce tissue homogeniser in the presence of PSG and trypanosomes were then isolated by ion-exchange purification as above.

4.2.3 Antibody-mediated attachment of parasites to macrophages

Peritoneal cells were washed from female CFLP mice in incomplete Eagles MEM and the approximate number of monocyte/macrophage lineage cells in the washings was determined by counting cells in the presence of 0.002 % Evans' blue solution. Macrophages were then plated out at an approximate density of 10^6/ml in complete MEM (cMEM; i.e. 84 ml incomplete MEM plus 15 ml heat-inactivated foetal calf serum and 1 ml of a 200 mM L-glutamine solution) into wells of a 24-well culture plate containing circular glass coverslips. The plates were incubated overnight (37°C, 5% CO_2) and then the medium was changed to wash away non-adherent cells.

Experiments were conducted using two VATs: GUTats 7.2 and 7.13. Slender and stumpy form parasites were isolated from the blood of infected female CFLP mice on days 3 and 6 of infection respectively. The VAT and morphological composition of the parasite populations were checked as previously (by indirect IFT and analysis of Giemsa-stained smears). Parasites were separated from the red blood cells as before. Trypanosomes (10^5/ml) and specific antibody diluted in cMEM were then added to the macrophage-containing wells in the absence of exogenous complement. At the end of a 30 min. incubation period (37°C, 5% CO_2), the medium was decanted from the wells and the coverslips were allowed to dry before addition of methanol for 10 mins. This fixed the macrophages which had adhered to the coverslips, and the coverslips were then placed in Giemsa's stain for 10 mins, washed, air dried and mounted face down on glass slides. The percentage of macrophages to which parasites had adhered was determined. Control wells contained identical numbers of trypanosomes of a different VAT.

4.3 RESULTS

4.3.1 Complement-mediated lysis

From Fig. 4.1 it can be seen clearly that lysis of bloodstream *T. brucei in vitro* is mediated by specific antibody. Negligible lysis of GUTat 7.13 occurred in the presence of GUPM 18.7. Parasites obtained from mice on the third day after infection (slender forms) were lysed very rapidly: > 50% were lysed in 10 mins and 95% were lysed within 45 mins. With the stumpy form population over 50 mins elapsed before 50% of the parasites were lysed and by the end of the experiment (90 mins) only 70% had been lysed. The large error bars in Fig. 4.1 are due to variability between individual experiments (see section 4.4).

In the presence of complement *in vitro* slender forms *T. brucei* were lysed approximately 5 times more rapidly than homologous stumpy forms. Fig. 4.2 shows percentage lysis plotted against time for slender, stumpy and mixed morphology populations in four replicate experiments using GUTat 7.2. A series of curves was obtained for each experiment showing that the rate of lysis of a trypanosome population increases with the proportion of slender forms in the population. The times to 50% lysis for each population in each of the four replicate experiments were derived from these graphs and are shown in Table 4.1, along with the accurate morphological composition of each population. These results show that slender forms were lysed between 2.5 and 15 times faster than stumpy forms in these experiments. The time to 50% lysis for each population in each experiment plotted against the percentage of slender forms in the particular population (Fig. 4.3) illustrates the relationship between morphological composition and rate of lysis. Linear regression analysis (see section 4.4) allows extrapolation of the results to show that a hypothetical 100% slender population would be lysed in approximately 9 mins whereas the

corresponding hypothetical 0% slender population would be lysed in approximately 66 mins *in vitro*, i.e. 7 times more slowly than the slender population.

When trypanosomes from the bloodstream and lymph nodes of mice on day 6 post-infection were incubated with specific antibody and complement the results shown in Fig. 4.4 were obtained. The morphological compositions of the four populations used in this experiment are given in Table 4.2 which shows that the bloodstream populations were predominantly stumpy and the lymph node populations were predominantly slender. As Fig. 4.4 illustrates the population expressing GUTat 7.2 that was derived from lymph nodes underwent very rapid lysis upon incubation with specific antibody plus complement: by t=10 mins 98% of the population had been lysed. The relatively high background level of parasite death (19% at t=0) was probably due to the rigours of the isolation procedure. In contrast, the rate of lysis of the stumpy form population was much slower: 50% of the parasites were lysed in 30 mins and 95% were lysed in 45 mins. Lysis of GUTat 7.13 parasites from lymph nodes and bloodstream in the presence of anti-GUTat 7.2 monoclonal antibody did not exceed 20% in the same 45 min. period. The reason for the 20-30% death rate among the control lymph node population is unclear. IFT did not detect any parasites expressing GUTat 7.2 in this population, so it is possible that there was a certain background mortality associated with isolation from lymph nodes, probably because of physical stress to the cells in the homogeniser.

4.3.2 Antibody-mediated attachment of parasites to macrophages

The attachment of trypanosomes to peritoneal macrophages was considerably enhanced by the presence of specific antibody. Four replicate experiments were conducted using GUTat 7.2 and specific

monoclonal antibody (Fig.4.5) and four experiments were conducted using GUTat 7.13 and a specific heat-inactivated antiserum (Fig. 4.6). In all eight experiments the presence of specific antibody caused an increase in attachment. Also, in seven of the eight experiments there was little apparent difference between the level of attachment of slender forms and that of stumpy forms in the presence of specific antibody.

When examining the results of the individual experiments it is apparent that there was variability between experiments in the extent of attachment in the absence or presence of specific antibody. For example, in experiment 1 (Fig. 4.5) the addition of specific antibody did not increase the extent of parasite attachment to macrophages above 18% for slender forms and 13% for stumpy forms; these values are comparable with the levels of attachment in the absence of the specific antibody in experiments 2-8. However, the underlying phenomenon was consistent: addition of specific antibody increased attachment of parasites to macrophages.

Only one experiment seriously challenged the finding that there is no difference between slender and stumpy forms in their antibody-mediated attachment to phagocytes. In the atypical experiment (experiment 5) the extent of attachment of slender forms to macrophages increased from 0% to 14% in the presence of specific antibody whereas the percentage of macrophages which had attached stumpy forms was greatly elevated (from 4% to 90%).

To analyse these results further two factor analysis of covariance was used to determine if antibody-mediated enhancement of attachment was significant and also if there was a significant difference between the antibody-mediated enhancement of attachment of slender forms compared

to stumpy forms. Each VAT was analysed separately and a summary of the calculations is given in Table 4.3. For both antigen types the presence of homologous antibody significantly increased the level of attachment of parasites to macrophages. For both antigen types there was no significant difference between the level of attachment of slender forms and stumpy forms. The experiments with GUTat 7.2 used homologous monoclonal antibody; those with GUTat 7.13 used a specific rabbit antiserum that had been heat-inactivated to preclude any lysis or C3b-mediated attachment. Therefore, the results observed were dependent only on the presence or absence of specific antibody. Differences in parasite antigen type or source of antibody had no effect.

Fig. 4.7 summarises the results shown in Figs 4.5 and 4.6. Variability between runs explains the large error bars in this figure, but in spite of this variability and the anomalous experiment 5, the mean values for attachment of slender and stumpy forms to macrophages in the presence of specific antibody are very similar. Using GUTat 7.2, 54% of macrophages had attached slender forms in the presence of specific antibody (shaded bar in column 2), and 47% had attached stumpy forms (shaded bar in column 3). Using GUTat 7.13, 71% of macrophages had attached slender forms in the presence of specific antibody (shaded bar in column 4), and 62% had attached stumpy forms (shaded bar in column 5).

The mean ± 2SE percentage of macrophages with attached parasites of both morphological forms together in the absence of specific antibody was 8.9 ± 5.6% (black bar in column 1) whereas the mean number of macrophages with parasites attached in the presence of specific antibody was 62.2 ± 14.8% (shaded bar in column 1). This pooled result

is valid because two factor analysis of covariance showed no difference between the results using slender and stumpy forms (compare the second pair of columns in this figure with the third, or the fourth with the fifth) and because similar results were obtained with both antigen types (compare second and/or third pair of columns with fourth and/or fifth pair).

4.4 DISCUSSION

Infections with *Trypanosoma brucei* stimulate production of large amounts of antibody of the IgM and IgG classes in mammalian hosts (see review by Mansfield, 1981). This increase in immunoglobulin synthesis and secretion is trypanosome-specific (Herbert *et al.*, 1980; Musoke *et al.* 1981). The specific antibody response is directed against the VSG (reviewed by Murray & Urquhart, 1977) which covers bloodstream *T. brucei* in a densely-packed layer (Vickerman, 1969) preventing access of external macromolecules such as antibody to any other surface components (Pearson *et al.*, 1980). Antibody produced in response to *T. brucei* infection mediates mechanisms which effect the clearance of the parasite from the bloodstream. The principal mechanisms are phagocytosis and complement-mediated lysis. The primary event in both these processes is recognition of antigenic epitopes by specific antibody molecules.

The parasite population as a whole survives the specific immune onslaught because some trypanosomes are expressing different VSG coats from that against which the immune response is directed. Thus, an antibody response developing against one antigen type in a population will have no effect on other VATs. Also, there will be no response to any VAT if the numbers of trypanosomes expressing that VAT are present at numbers below the threshold level of detection by the immune system

(Seed & Sechelski, 1988).

Resistance to trypanosomiasis correlates with the ability of the host to control the level of parasitaemia, as reviewed in chapter 1, and the major component of control of parasitaemia is killing of parasites and their clearance from the bloodstream. Slender form parasites undergoing division are responsible for increases in parasitaemia. Therefore, the results presented in this chapter suggest that the higher susceptibility of slender forms to complement-mediated lysis provides a mechanism by which parasitaemia growth can be curtailed by the developing antibody response. At the same time, the differentiation of some parasites to stumpy forms (which occurs at all times during a wave of infection) contributes to a reduction in the overall rate of growth of the population. Remission is achieved by clearance of both forms.

Immunological clearance of trypanosomes is thought to be mediated primarily by uptake of opsonised parasites by phagocytic cells in the liver and spleen. MacAskill *et al.* (1981) reported that they found no evidence for intravascular lysis in immune mice and therefore assumed that live trypanosomes were being cleared from the circulation and subsequently killed by macrophages. However, lysis has been observed in the tailblood of immune mice (Vickerman & Barry, 1982) thus leaving open the question of whether trypanosomes are killed before clearance from the circulation, or afterwards, or both. The importance of a particular effector mechanism may depend on the host species.

Results presented in this chapter show that differential lysis of bloodstream parasites does occur *in vitro*. There was a reproducible, significant difference between the rates of complement-mediated lysis of slender and stumpy form *T. b. rhodesiense*. The quantitative

difference varied between replicate experiments but the phenomenon was constant. If such differential lysis occurs *in vivo* it will contribute to the drop in absolute number of slender forms that occurs at the parasitaemic peak before a decrease in the total population size is observed (section 2.2).

In Fig. 4.1. the very large error bars in the curve representing the predominantly stumpy form population arise from two sources. The first is the actual proportion of stumpy forms as established by differential morphological counting of Giemsa-stained smears. Although stumpy forms predominated over slender forms in all day 6 post-infection populations, the exact proportion varied between the four experiments (from 43 to 77%). The second source of error was the variability in the rates of the lysis reactions between the four experiments. Rate of lysis varies with temperature. These experiments were carried out at room temperature which varied between 19 and 23°C. This second factor must also have contributed to the error bars in the graphs representing the predominantly slender form population.

In Fig. 4.3 the relationship between the proportion of slender forms in a population and the rate at which that population is lysed by specific antibody plus complement *in vitro* is described by a linear regression line. However, this line is not necessarily the best mathematical descriptor of the relationship between the two parameters. From this particular set of results it seems possible that an exponentially decaying curve might be a more accurate descriptor of the relationship: though, only one point on the graph suggests this. Many repetitions of this experiment would be required to establish the true nature of the relationship between "slenderness" and rate of lysis. The simple linear regression line shown here simply illustrates that the two parameters are related, and that this simplest

relationship is a reasonable possibility.

In order to compare the susceptibility to lysis of slender and stumpy forms *in vitro*, parasite populations in which either form predominated were required. It was not possible to purify either form from a mixed population (see Chapter 2) so populations from different days of infection were used, making use of the knowledge of how morphological prevalences change with time. A possible objection to this is that events occurring in the bloodstream alter the stumpy form parasites in some way that affects other properties as well as their gross morphology to the extent that day 3 and day 6 parasites cannot be compared. This objection was overruled by using slender and stumpy form parasites obtained on the same day of infection from bloodstream and lymph nodes of the same animal. Although the use of parasites from extravascular compartments itself may allow that intrinsic differences exist, this control experiment (the results of which are shown in Fig. 4.4) had the advantage of showing that the higher susceptibility of slender forms to lysis was a consistent property.

In contrast to the findings with lysis *in vitro*, there was no difference in the ability of phagocytic cells to bind slender and stumpy parasites in the presence of specific antibody. Specific antibody significantly increased the extent of attachment of trypanosomes to phagocytic cells suggesting that all VSG-bearing trypanosomes bind specific antibodies which mediate immune effector functions, but that the mechanism by which slender forms are more susceptible to lysis than stumpy forms is peculiar to this particular effector mechanism and is not an intrinsic part of the way in which trypanosomes and specific antibody interact.

Figs 4.5 and 4.6 both show data obtained in four experiments. It can be seen, however, that there was great variability between results obtained in each experiment although the trend was consistent. Part of this variability is attributable, as mentioned above, to the non-identical proportions of stumpy forms present in the day 6 populations used. The phagocytic cells used in these experiments were obtained by flushing the peritoneal cavities of outbred CFLP mice. As well as differences in Fc receptor (FcR) expression attributable to genetic variability between mice, it is possible that animals from different batches at different times had various concurrent viral infections which might cause differences in the degrees of activation of their macrophages.

It is interesting to speculate on reasons for the greater susceptibility of slender forms to lysis mediated by antibody plus complement. Complement-mediated lysis requires assembly at and insertion into a membrane of several macromolecular components collectively termed the membrane attack complex. The vast expanse of *T. brucei* membrane and flagellum, however, is completely covered with VSG and therefore is inaccessible to the pore-forming proteins which might be recruited to the parasite surface as a result of fixation of complement by the specific antibody-VSG complexes. The only known region of *T. brucei* where the layer of VSG is interrupted is in the flagellar pocket: this is where receptor-mediated endocytosis of proteins occurs and receptor proteins penetrate the VSG layer. Webster & Grab (1988) have demonstrated internalisation of VSG by labelling of fixed preparations with antibody. It is possible that endocytosis may allow access of the membrane attack complex to phagosomes bounded by plasma membrane, and that this may cause serious disruption of the trypanosome leading to lysis. Since slender forms divide rapidly it

might be expected that slender forms have a higher rate of endocytosis than stumpy forms; this is not the case, however (Langreth & Balber, 1975; Vickerman, 1985), so that no correlation between the rates of endocytosis and lysis can be identified.

The results obtained *in vitro* can be used to construct a model for events occurring *in vivo*. Peak parasitaemia coincides with the appearance of measurable specific antibody titres (Barry & Emery, 1984). Since antibody- and macrophage-mediated clearance are known to be important *in vivo*, any preferential clearance of slender forms by this route would significantly alter the morphological profile of the parasitaemia at the peak. The *in vitro* assays of attachment of opsonised trypanosomes to phagocytic cells described in this chapter did not, however, show any preferential destruction of slender or stumpy forms. Antibody significantly enhanced attachment of parasites of both morphological forms to macrophages. This occurred in the absence of complement components indicating that attachment to macrophages mediated by the Fc fragment of immunoglobulins was occurring in this system, although MacAskill *et al.* (1980) reported that C3b was the opsonin responsible for attachment of trypanosomes to macrophages. Thus, macrophage uptake of opsonised parasites is probably responsible *in vivo* for much of the sudden drop in numbers of parasites occurring at peak parasitaemia, irrespective of their morphological forms.

The results obtained from the complement-mediated lysis experiments suggest that preferential lysis of slender forms occurs *in vivo*. When lysis has been observed previously (Vickerman & Barry, 1982) in tail-blood from immunised animals, it occurred in the first minute or so after challenge. Such a rapid phenomenon is very difficult to quantify or study experimentally and this may explain why lysis has not been considered to be an important immune clearance mechanism in

the past.

Table 4.1. Percentage of slender forms in each experimental population and the corresponding time to 50% lysis in the presence of specific antibody plus complement.

Experiment (Fig.)	Slender:stumpy ratio	% slender forms from Giemsa-stained smears	Time to 50% lysis (mins)
1 (4.2a)	1:0	99.0	9
	3:1	38.6	16
	1:1	41.3	30
	1:3	14.3	40
	0:1	13.1	48
2 (4.2b)	1:0	93.0	14
	3:1	72.1	20
	1:1	51.1	25
	1:3	30.2	28
	0:1	9.3	35
3 (4.2c)	1:0	100.0	10
	3:1	60.0	17
	1:1	54.0	20
	1:3	10.0	69
	0:1	8.0	150
4 (4.2d)	1:0	100.0	8
	3:1	68.0	15
	1:1	39.4	69
	1:3	29.0	56
	0:1	0.0	51

Table 4.2. Morphological compositions of the parasite populations derived from bloodstream and lymph nodes of mice on day 6 post-infection. LN = lymph node-derived parasites; BS = bloodstream-derived parasites.

	GUTat 7.2		GUTat 7.13	
	LN	BS	LN	BS
% slender forms	78.0	15.5	92.0	7.0
% intermediate forms	21.0	31.5	6.0	19.0
% stumpy forms	1.0	53.0	2.0	74.0

Table 4.3. Summary of two factor analysis of covariance.

GUTat 7.2

Source	Sum of squares	df	Mean squares	F
Factor A	6967.24	1	6967.24	14.10
Factor B	37.33	1	37.33	0.08
Interaction	75.52	1	75.52	0.15
Residual	5931.63	12	494.30	-
Total	13011.72	15		

GUTat 7.13

Source	Sum of squares	df	Mean squares	F
Factor A	16892.43	1	16892.43	30.90
Factor B	295.24	1	295.24	0.54
Interaction	44.92	1	44.92	0.08
Residual	6559.84	12	546.65	-
Total	23792.427	15		

	GUTat 7.2		GUTat 7.13	
	$F_{(1,12)}$	P	$F_{(1,12)}$	P
	($F_{0.01\ (1,12)}$ = 9.3302 from tables)			
with/without antibody	14.10	<0.01	30.90	<0.01
slender/stumpy	0.08	>0.05	0.54	>0.05

Percentage lysis against time in predominantly slender and predominantly stumpy form populations

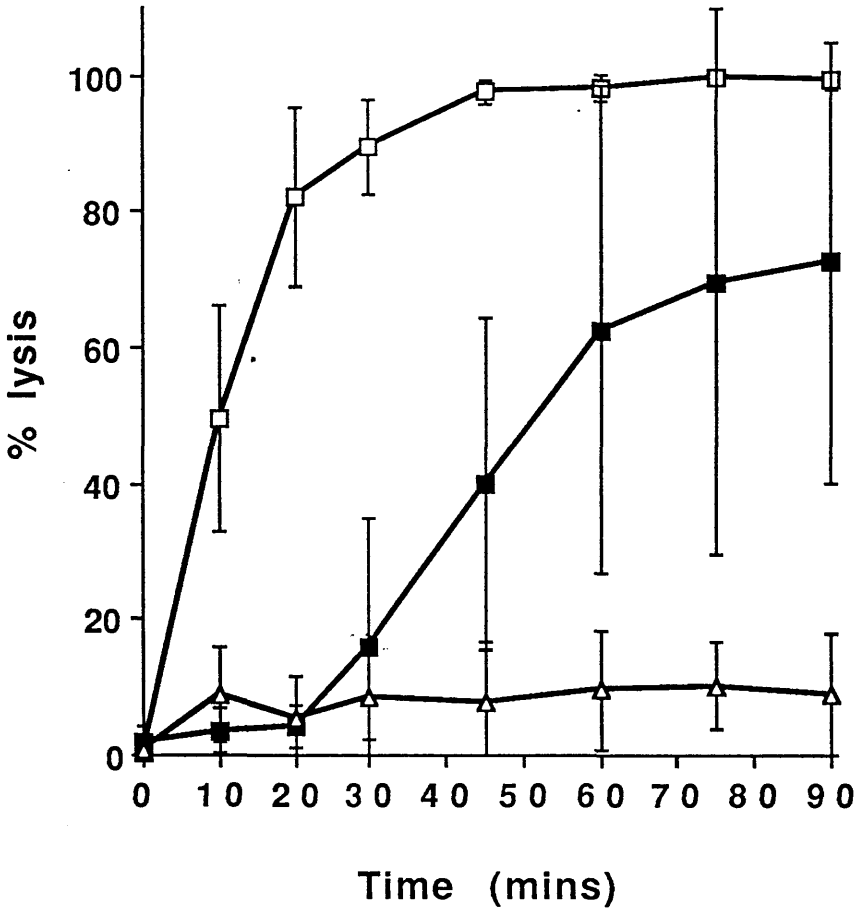


Fig. 4.1. Percentage lysis against time in predominantly slender and predominantly stumpy populations of *Trypanosoma brucei* in the presence of specific antibody and complement (mean ± 2SE percentage of parasites lysed with time for four replicate experiments).

□ Predominantly slender population expressing GUTat 7.2 in the presence of homologous specific antibody (GUPM 18.7).

■ Predominantly stumpy population expressing GUTat 7.2 in the presence of homologous specific antibody (GUPM 18.7).

△ Predominantly slender population expressing GUTat 7.13 in the presence of heterologous antibody (GUPM 18.7).

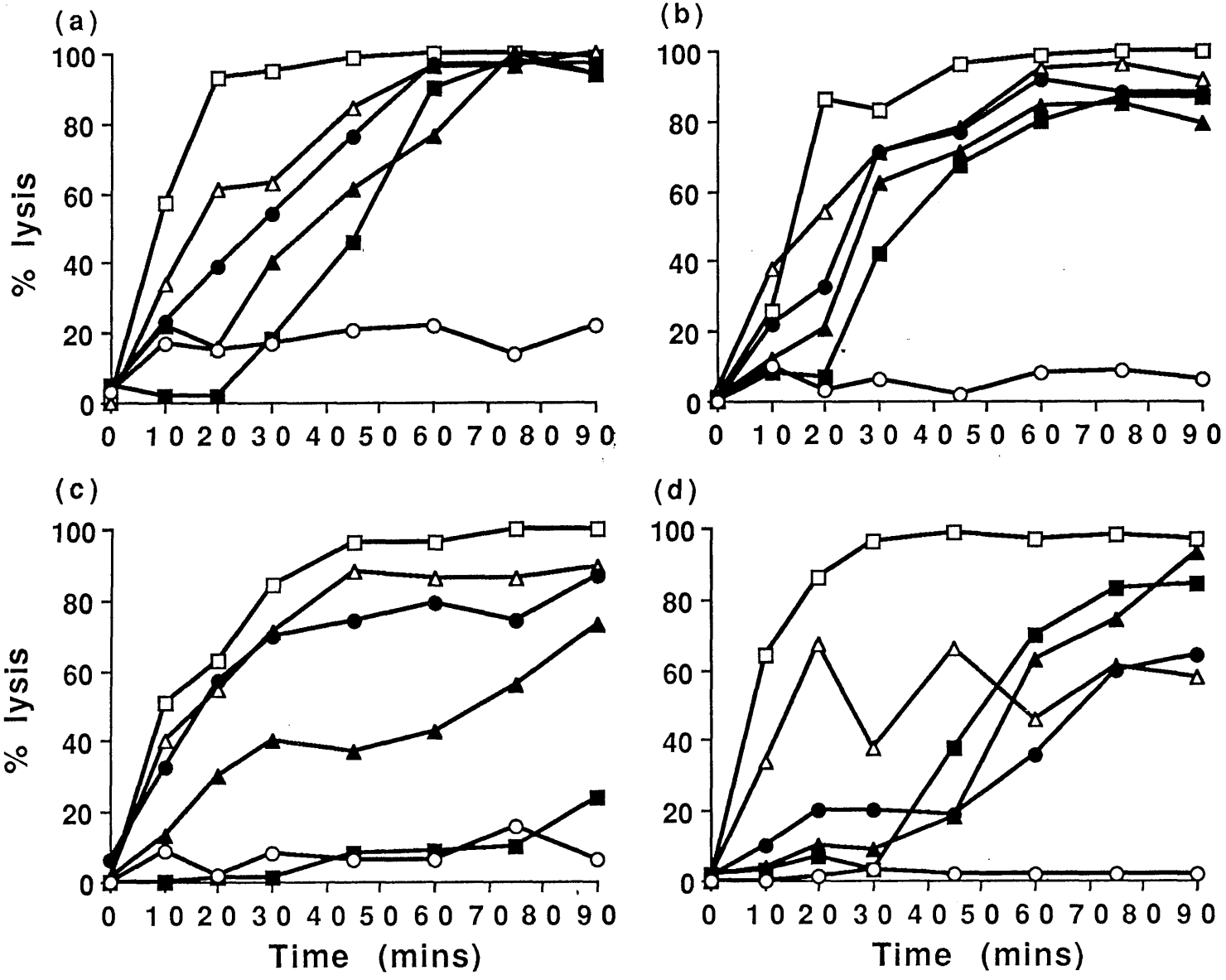

(a)
(b)
(c)
(d)
% lysis
% lysis
0
20
40
60
80
100
0 10 20 30 40 50 60 70 80 90
Time (mins)
Time (mins)

Fig. 4.2. Lysis of *T. brucei* by specific antibody and complement.

Percentage of parasites lysed with time for slender, mixed morphology, stumpy and control populations. The four experiments the results of which are shown in (a) - (d) were conducted on different days using parasites expressing GUTat 7.2 in the presence of specific antibody (GUPM 18.7). The control populations were slender form parasites expressing GUTat 7.13.

□ Predominantly slender form population expressing GUTat 7.2 isolated from mice on day 3 post-infection.

△ Mixture of slender and stumpy populations in the ratio 3:1.

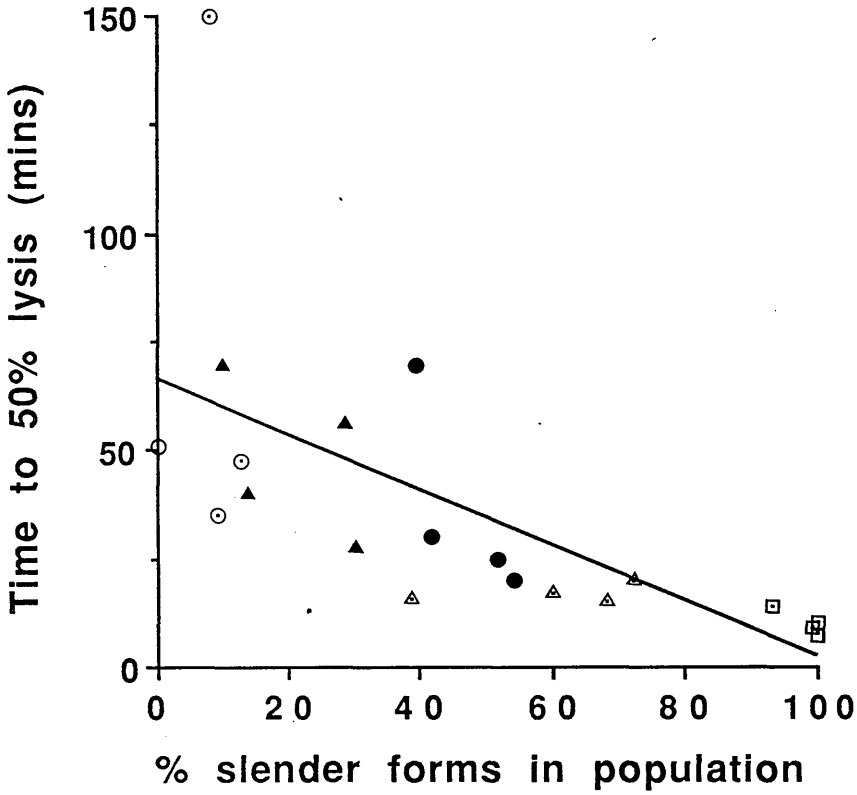
● Mixture of slender and stumpy populations in the ratio 1:1.

▲ Mixture of slender and stumpy populations in the ratio 1:3

■ Predominantly stumpy form population expressing GUTat 7.2 isolated from mice on day 6 post-infection.

○ Control in which parasites were slender forms expressing GUTat 7.13.

Relationship of the time to 50% lysis to the morphology of the parasite population



Percentage lysis of parasites derived from the bloodstream and lymphnodes of an infected mouse on day 6 post-infection

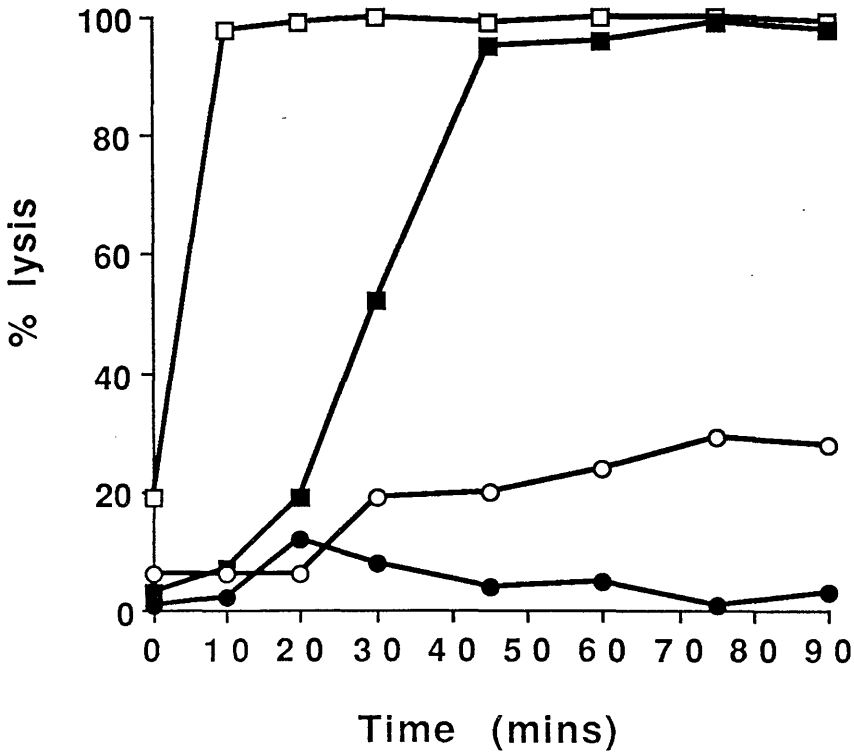


Fig. 4.3. Relationship of the time to 50% lysis to the morphology of the parasite population.

This graph shows the pooled results from the four replicate experiments shown in Fig. 4.2 and Table 4.1. There are five different sets of symbols on this graph, each symbol representing one morphological population as follows:

□ Predominantly slender form population only.

△ Slender:stumpy 3:1.

● Slender:stumpy 1:1.

▲ Slender:stumpy 1:3.

○ Predominantly stumpy form population only.

Fig. 4.4. Percentage lysis of parasites derived from the bloodstream or lymph nodes of mice on day 6 post-infection.

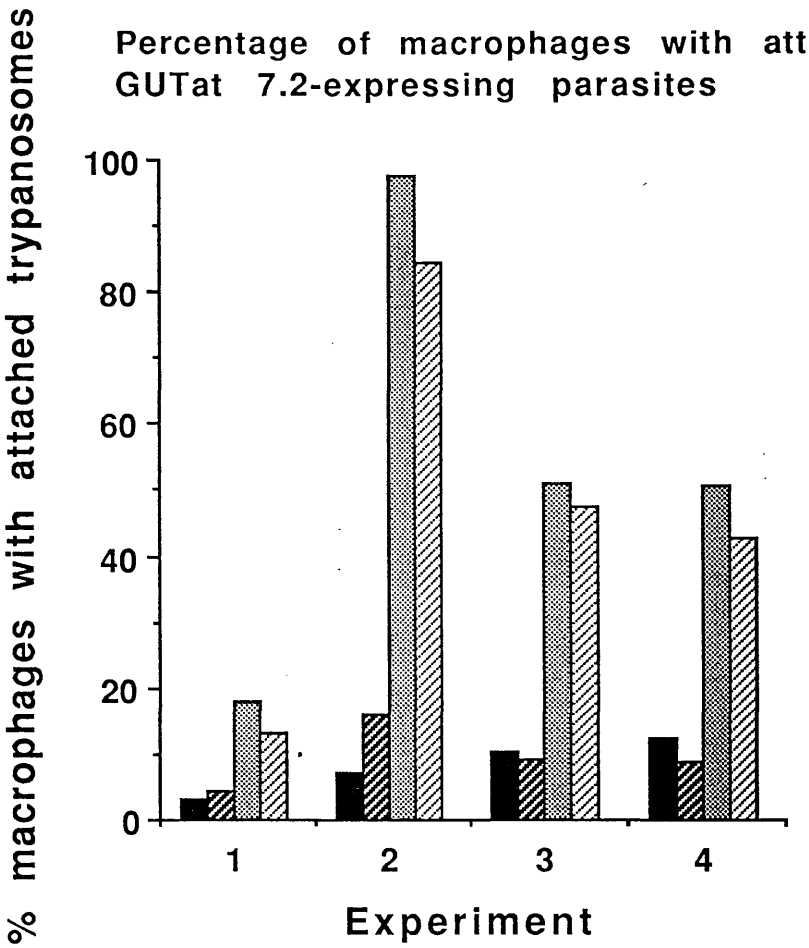
Bloodstream and lymph node populations of trypanosomes were isolated from mice infected with each of two VATs (GUTat 7.2 and GUTat 7.13) on day 6 post-infection. At this time bloodstream trypanosomes are predominantly stumpy forms whereas extravascular populations are composed predominantly of slender forms. The trypanosomes were incubated with antibody specific for GUTat 7.2 in the presence of complement.

□ Predominantly slender form trypanosomes from lymph nodes of mice infected with GUTat 7.2.

■ Predominantly stumpy form trypanosomes from the bloodstream of mice infected with GUTat 7.2.

○ Predominantly slender form trypanosomes from lymph nodes of mice infected with GUTat 7.13.

● Predominantly stumpy form trypanosomes from the bloodstream of mice infected with GUTat 7.13.


Percentage of macrophages with attached GUTat 7.2-expressing parasites
% macrophages with attached trypanosomes
100
80
60
40
20
0
1
2
3
4
Experiment

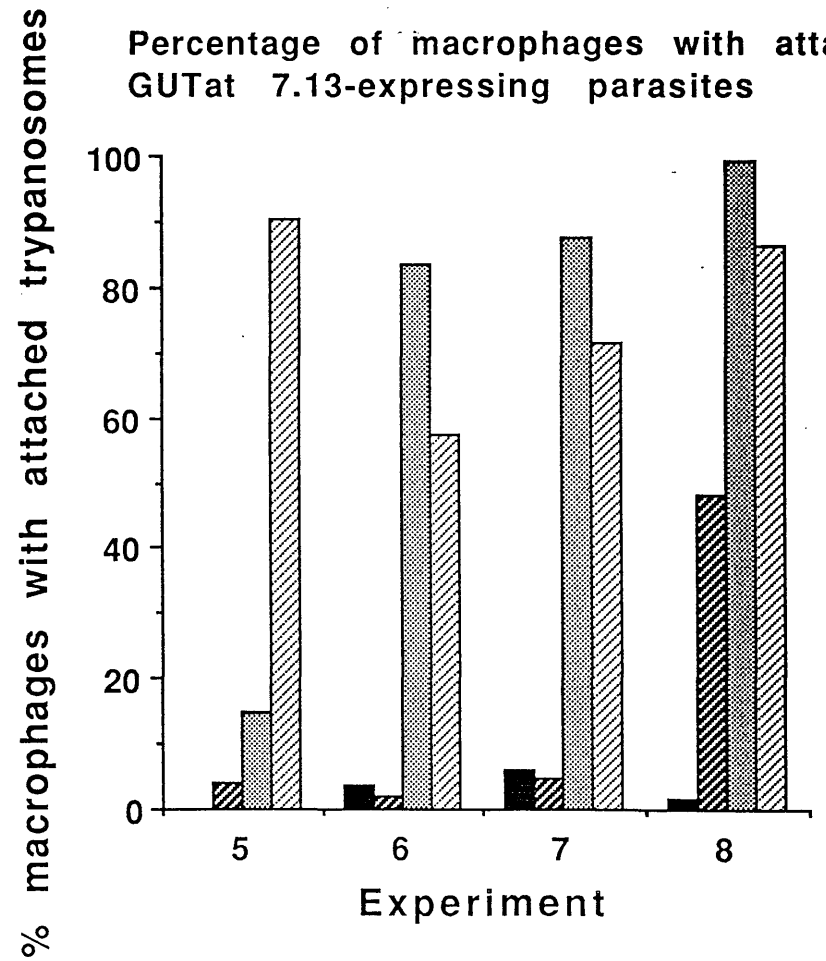

Percentage of macrophages with attached GUTat 7.13-expressing parasites
% macrophages with attached trypanosomes
100
80
60
40
20
0
5
6
7
8
Experiment

Fig. 4.5. Percentage of macrophages with attached GUTat 7.2 trypanosomes in the presence of specific monoclonal antibody GUPM 18.7.

Predominantly slender forms in the absence of specific antibody.

Predominantly stumpy forms in the absence of specific antibody.

Predominantly slender forms in the presence of specific antibody.

Predominantly stumpy forms in the presence of specific antibody.

Fig. 4.6. Percentage of macrophages with attached GUTat 7.13 trypanosomes in the presence of specific rabbit antiserum.

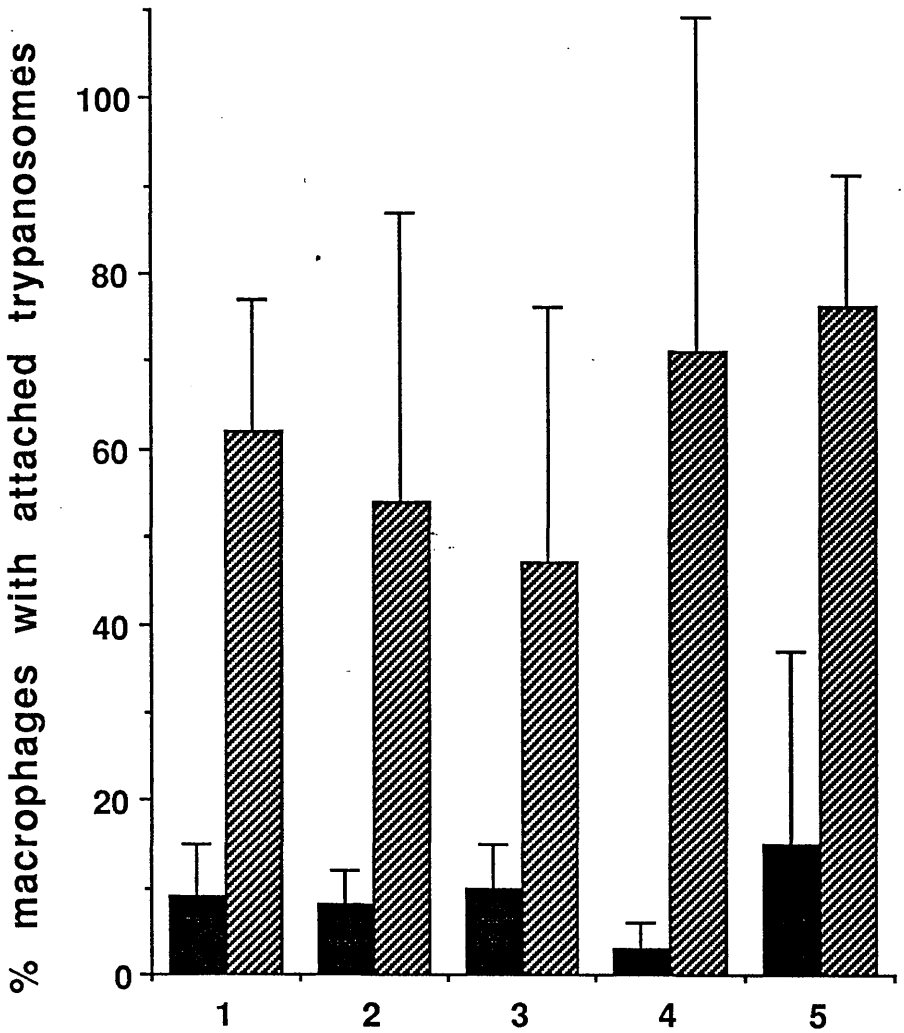
Predominantly slender forms in the absence of specific antibody.

Predominantly stumpy forms in the absence of specific antibody.

Predominantly slender forms in the presence of specific antibody.

Predominantly stumpy forms in the presence of specific antibody.

Percentage attachment to macrophages of parasites expressing GUTats 7.2 and 7.13 in the presence of specific homologous antibody



facing page 116

Fig. 4.7. Mean ± 2SE percentage attachment to macrophages of parasites expressing GUTat 7.2 and GUTat 7.13 in the presence of their respective homologous antibodies.

This figure shows the combined results from Figs 4.5 and 4.6. The pairs of bars represent the following:

1 pooled mean ± 2SE percentage attachment to macrophages of slender plus stumpy forms of both antigen types in the presence or absence of specific antibody;

2 mean ± 2SE percentage attachment to macrophages of slender forms expressing GUTat 7.2;

3 mean ± 2SE percentage attachment to macrophages of stumpy forms expressing GUTat 7.2;

4 mean ± 2Se percentage attachment to macrophages of slender forms expressing GUTat 7.13;

5 mean ± 2SE percentage attachment to macrophages of stumpy forms expressing GUTat 7.13.

 in absence of specific antibody

 in presence of specific antibody

Chapter 5

DETECTION OF SPECIFIC ANTIBODY IN THE SERUM OF *T. BRUCEI*-INFECTED MICE AFTER DRUG TREATMENT

5.1 INTRODUCTION

It has been claimed that slender form parasites do not induce an immune response in mice whereas stumpy forms do. The evidence backing this assertion is the supposedly low antibody titre produced against virulent, monomorphic (slender form only) parasites (Black, Hewett & Sendashonga, 1982) in these hosts. Sendashonga & Black (1982) further suggested that the lack of antibody response against slender forms is related to the method of presentation of the VSG to the host immune system. They suggested that the degeneration of stumpy forms whose half-life is approximately 24-36 h leads to release of membrane fragments on which VSG in its native form is expressed, and that it is VSG on such fragments that is responsible for stimulating a specific immune response, rather than VSG on intact parasites or soluble VSG. However, there are data that conflict with the results of Black *et al.* (1982) and show that monomorphic parasites do elicit specific antibody responses in rats (Zahalsky and Weinberg, 1976) and possibly in rabbits, although parasitaemias in rabbits were very low (Scott, 1987). This apparent conflict may be due to methodological differences between the reports or it may indicate that serum antibody levels are an intrinsically unreliable indicator of host immune responses. It seems unlikely that a glycoprotein molecule such as trypanosome VSG presented on the parasite surface as a vast array of epitopes should not stimulate a strong specific antibody response in the mammalian host. Variable antigen is the most abundant protein in the trypanosome cell (Vickerman & Barry, 1982).

A possible explanation for the unreliability of detection of serum

antibody is that African trypanosomes may act as an antibody "sink", stimulating a specific antibody response and then binding antibody in sufficient quantities to lower the levels of free antibody in the serum but at concentrations per individual cell that are insufficient for any antibody-mediated immune effector mechanisms to proceed. The rapid chemotherapeutic removal of parasites, if they do act as a sink, should allow detection of antibody newly-secreted by plasma cells at the time of drug cure. That is, an increase in specific serum antibody titre would be detected and this increase would occur too quickly to be due to induction of an immune response by the dead and dying parasites. This possibility was tested by the experiments described in this chapter. VAT-specific serum antibody levels were measured in mice in which infections were drug-cured and compared with those from mice that naturally resolved infections. Serum antibody responses to infection were monitored using agglutination which is a very sensitive test for the presence of specific antibody, and complement-dependent lysis which tests for a specific functional activity of the antibody.

5.2 MATERIALS AND METHODS

5.2.1 Trypanosomes and antisera

Two pleomorphic parasite lines expressing GUTats 7.2 and 7.13 (described in Chapter 2) were used in these experiments. The same rabbit antisera and monoclonal antibodies were also used. The optimum dilutions for the use of each antiserum or ascitic fluid were established in preliminary experiments (results not shown). Where required, antisera were heat-inactivated (to destroy complement components) by incubation at 56^{o}C for 30 mins.

5.2.2 Experimental design

Serum samples from infected and cured, and from infected but not cured

groups of female BALB/c mice were compared. Duplicate experiments were conducted using the two VATs. Mice (6 per group) were inoculated intraperitoneally with 10^5 trypanosomes each and cured 3 days later. The control, uncured mice naturally entered remission on day 7 or 8 of infection (see section 2.2). Serum samples were collected from the mice immediately before cure, 8 h after cure and thereafter at approximately 24 h intervals. These samples were pooled for each group at each time-point and tested for specific antibody activity against trypanosomes of homologous VAT in agglutination and lysis assays.

5.2.3 Cure of *T. brucei* infections with SHAM-glycerol

The cure regimen consisted of a first subcurative dose of 30 μl 0.5M salicylhydroxamic acid (SHAM) and 0.1 g glycerol per mouse to reduce the parasitaemia to approximately 10^6 parasites/ml blood, followed 2 h later by a full curative dose, 150 μl SHAM plus 0.1 g glycerol (Van Der Meer *et al.*, 1979). SHAM is a trypanostatic, rather than a trypanocidal compound. Complete inhibition of L-glycerol-3-phosphate oxidase with SHAM halves the intra-trypanosomal ATP levels, and reduces motility. In the presence of glycerol motility of the trypanosomes ceases completely within approximately 5 mins. Accumulation of large numbers of dead parasites in the blood vessels of the kidneys may cause fatal organ damage, that is why the parasitaemia was reduced by a subcurative dose before the full cure. SHAM itself has toxic side effects on mammals due to depression of the CNS: the effective dose is very close to the LD_{50} (Opperdoes *et al.*, 1976).

5.2.4 Agglutination assay

Parasites were separated from the blood on DEAE-cellulose columns (Lanham & Godfrey, 1970) and concentrated by centrifugation to an approximate density of 3 x 10^8/ml in PSG (pH 8). The parasites were

In all experiments, parasites expressing a heterologus VAT were used as negative controls.

mixed in a 1:1 ratio with serum samples diluted in PSG such that the final serum concentrations in the assay were doubling dilutions in the range 1/10 to 1/10240. The mixtures were incubated at room temperature for 30 min. The highest serum dilution giving "1+" agglutination was recorded (Cunningham & Vickerman, 1962).*

5.2.5 Lysis assay

This was conducted essentially as described in section 4.2.3. Blood containing approximately 10^8 parasites/ml was mixed with test serum samples diluted in fresh guinea pig serum (a source of complement) such that the final test serum concentrations in the assay were 1/5, 1/10 and so on to 1/2560. The mixtures were incubated for 2 h at room temperature. The proportion of dead parasites was counted at the end of this time and the highest serum dilution giving > 50% lysis was recorded.

In both the agglutination and lysis experiments trypanosomes expressing a heterologous VAT were used as negative controls. In the lysis experiments, guinea pig serum without specific antiserum was used to ensure that the lysis required the presence of specific antibody and could not occur in the presence of complement alone.

5.3 RESULTS

The results of the agglutination and lysis assays are shown Table 5.1 and transformed data ($\log_2$ of reciprocal of highest test serum dilution giving 1+ agglutination or > 50% lysis) are plotted in Figs 5.1 (GUTat 7.13) and 5.2 (GUTat 7.2).

5.3.1 Agglutination assay using serum from mice infected with GUTat 7.13

Immediately before cure (t=0 in Fig. 5.1a) no agglutination was

detected in serum samples from either group (infected then cured experimental group or infected but not cured control group). The test group were cured with SHAM-glycerol and serum samples were taken 8 h later. An agglutinating titre of 1/40 was detected in the cured group compared with 1/20 in the non-cured group. At 24 h after cure the respective values were 1/80 and 1/40, and at 48 h after cure they were 1/640 and 1/80. By day 3 (72 h) after cure) the agglutinating titre in the cured group had soared to 1/5120 while the titre in the non-cured group was 1/640. On day 6 after cure (144 h) the agglutinating titre of both groups was 1/640.

5.3.2 Lysis assay using serum from mice infected with GUTat 7.13

When the same serum samples were tested in a lysis assay no lysis was detected in either group until day 3 after cure (Fig. 5.1b) when a lytic titre of 1/5 was detected in the cured group (but no lysis was detected in the non-cured group). By day 6 after cure a lytic titre of 1/5 was detectable in the serum samples from both groups of mice.

5.3.3 Agglutination assay using serum from mice infected with GUTat 7.2

No agglutination was detected in serum samples from either group before cure or 8 h after cure (Fig. 5.2a). Three days after cure the agglutinating titre in the cured group was 1/2560 compared with 1/40 in the non-cured group, and on day 4 the picture was similar; 1/1280 compared with 1/80. By day 6 after cure both groups had an agglutinating titre of 1/1280 and on day 7 the titre in the cured group had increased to 1/2560.

5.3.4 Lysis assay using serum from mice infected with GUTat 7.2

No lysis was detected in either group until day 7 after cure (Fig. 5.2b) at which time the titre in the cured group was 1/10 and the

titre in the non-cured group was 1/5.

Thus, the results of both assays show the same pattern with both VATs. Antibody titres were higher, earlier in the cured group than in the non-cured group. In each experiment the same pooled sera were tested in both assays. It can be seen that agglutinating titres could be detected far more readily than lytic titres. This confirms that agglutination is approximately 2^7 more sensitive a test of serum antibody titre than lysis. It also indicates that trypanosomes can have significant amounts of antibody bound to them, enough for agglutination to occur, but that a greater amount of bound antibody is required for lysis to occur.

5.4 DISCUSSION

Specific antibody became detectable by agglutination assay in the serum of mice infected with GUTat 7.13 within a few hours of SHAM-glycerol cure. No specific antibody could be detected in the serum of non-cured control mice at the same time-point. Until day 6 the serum titres in the cured group were consistently higher than in the non-cured group. The results obtained with mice infected with GUTat 7.2 were similar, although no antibody was detected in the serum 8 h after cure. By day 6 the specific agglutinating titres of sera from both cured and non-cured groups of mice infected with either VAT were the same, showing that a specific serum antibody titre did develop in the non-cured group, but that this occurred over a longer period of time than in the cured group.

Two possible explanations can be offered for these results. Either trypanosomes act as an antibody sink, or dead (in this case drug-killed) trypanosomes are more immunogenic than live ones. The sharp

rise in agglutinating titre observed on day 3 post-cure occurs too rapidly to be due to stimulation of antibody production from plasma cells. This indicates that the first explanation is more likely. Most probably, the drug treatment killed and disrupted the parasites. The dead parasites and any debris such as VSG molecules or membrane fragments that were shed were aggregated by the attached antibody and cleared by splenic and hepatic macrophages. At the same time, specific antibody secretion from plasma cells, stimulated during the first 3 days of infection, continued at the same rate so that large quantities of specific antibody were secreted into the serum. Since no antigen was present to soak up the antibody, the antibody became detectable as a high serum titre *in vivo*.

In the non-cured groups antibody titres became detectable soon after their appearance in the cured groups, but at a consistently lower level until about days 6 and 7. This correlates with the time taken for a primary immune response to develop. Thus in non-cured mice the parasitaemia peaks at around day 6 and parasites are cleared from the bloodstream, so that parasitaemic remission occurs.

Since slender forms divide and stumpy forms do not, the former clearly have the potential to act as a larger sink. It is possible to envisage the following events. During the rising phase of a parasitaemia when slender form division is causing exponential parasite growth there is a vast amount of VSG available to which specific antibody may bind such that the detectable free serum antibody titre is markedly reduced. At the same time, individual parasites are binding insufficient antibody to cause damage, therefore significant clearance does not occur. As the parasitaemia advances, B cell proliferation and differentiation to plasma cells continues and the quantity of antibody

produced increases. Meanwhile, parasite differentiation to stumpy forms occurs and the parasitaemia begins to level off. Thus antibody reaches its effective titre relative to parasite density - and therefore antigen (VSG) density - at the time of peak parasitaemia and so immune mediated clearance of parasites occurs.

Following this scheme, infections with very rapidly dividing monomorphic parasites outstrip the antibody response so that the serum antibody titre never reaches the critical level necessary for immune clearance mechanisms to operate within the short survival time of infected mice. This may explain the inability of Black and colleagues to detect specific antibody in the serum of mice infected with monomorphic parasites.

There is evidence supporting this view. Infection of naive rats with a highly acute (monomorphic) parasite line leads to death at the first peak of infection and no phagocytic clearance is observed, whereas more chronic infections are accompanied by antibody-mediated phagocytic clearance (MacAskill *et al.*, 1981). These authors concluded that the acute infections they described were fatal because the host could not achieve effective levels of circulating antibody due to the very rapid replication rate of monomorphic strains. A different view of the interaction of monomorphic lines and the host immune system, that of Black, Hewett & Sendashonga (1982) and Sendashonga & Black (1982), has been described in section 5.1. As well as suggesting that release of membrane fragments bearing VSG in its native form by senescent stumpy forms is necessary for induction of an immune response, they propose that the failure of monomorphic parasites to stimulate antibody production is due to their inability to stimulate B cells (Sendashonga & Black, 1986), presumably because of lack of senescence of monomorphic parasites. In direct contradiction to this

Zahalsky & Weinberg (1976) reported an IgM agglutinating antibody titre of 1/4 in rats 2 days after infection with 10^5 parasites of a monomorphic laboratory strain of *T. brucei* (EATRO 691A). The titre rose sharply: 1/8 on day 3 post-infection and 1/64 on day 4. The background level of agglutinating activity in normal uninfected rat serum was 1/2 in these experiments.

As stated in section 5.1, VSG is highly immunogenic and it would be surprising if it did not stimulate strong specific antibody responses, no matter how it is presented to the host immune system. Indeed, specific IgM responses have also been detected within a few days of experimental infection of rabbits with *T. b. gambiense* (Seed *et al.*, 1969), of cattle with *T. congolense* and *T. vivax* (Luckins, 1976) and of cattle with *T. brucei* (Musoke *et al.*, 1981). A humoral response to metacyclic VATs is detectable 2-3 days after infection of goats by tsetse fly (Barry, Emery & Moloo, 1980). In human sleeping sickness high levels of IgM are found consistently in serum and in the cerebrospinal fluid (Houba & Allison, 1966). Antibody detected in all these cases, and particularly in the experimental infections using serially passaged predominantly slender from populations, would probably be stimulated by intact slender form parasites as well as senescent stumpy forms.

Black and colleagues explained the lack of host antibody response to infection with monomorphic parasites in terms of a block in antibody production by B cells: increased numbers of nucleated spleen cells were observed in such infections but there was no accompanying increase in detectable immunoglobulin secretion (Sendashonga & Black, 1982) and no immunoglobulin was produced after fusion of these spleen cells with mouse myeloma cells. This result must be viewed in the

context of other findings from that research group. Newson, Mahan & Black (1990) showed that antibody production (synthesis and secretion) occurred in the spleens of trypanosome-infected mice of a susceptible strain, although earlier work (Black *et al.*, 1986) had concluded that susceptible animals succumbed to infection because even though they had normal numbers of antibody synthesising plasma cells, secretion of antibody was blocked by some unknown mechanism.

Two mechanisms of B cell tolerance may cause apparent unresponsiveness to a large repetitive antigen as trypanosome-bound VSG. One is the effector cell blockage (Schrader & Nossal, 1974) in which specific antibody production is down-regulated by the binding of multivalent antigen to the membrane immunoglobulin receptors on B cells. The second is the so-called "treadmill tolerance" in which the presence of high concentrations of poorly degraded antigen leads to the ingestion of immune complexes by phagocytic cells, followed by release of the non-degraded antigen. This antigen then binds more antibody molecules in the serum and prevents detection of the antibody response, even though it exists.

The effects of drug cure on serum IgM levels in African trypanosome infections have been determined in several experimental systems. For example, Berenil treatment of *T. brucei* infections of cattle led to reduction of macroglobulinaemia (Luckins, 1976). Zahalsky & Weinberg (1976), however, reported that IgM levels in rats were sustained for about 28 days after Berenil treatment of a *T. brucei* infection. Black *et al.* (1986) reported that the block in antibody secretion observed in susceptible mouse strains was removed within 24 h of elimination of trypanosomes by Berenil treatment. These facets of the relationship of drug treatment to detection of antibody titres are not relevant to the discussion of the experiments described in this chapter because the

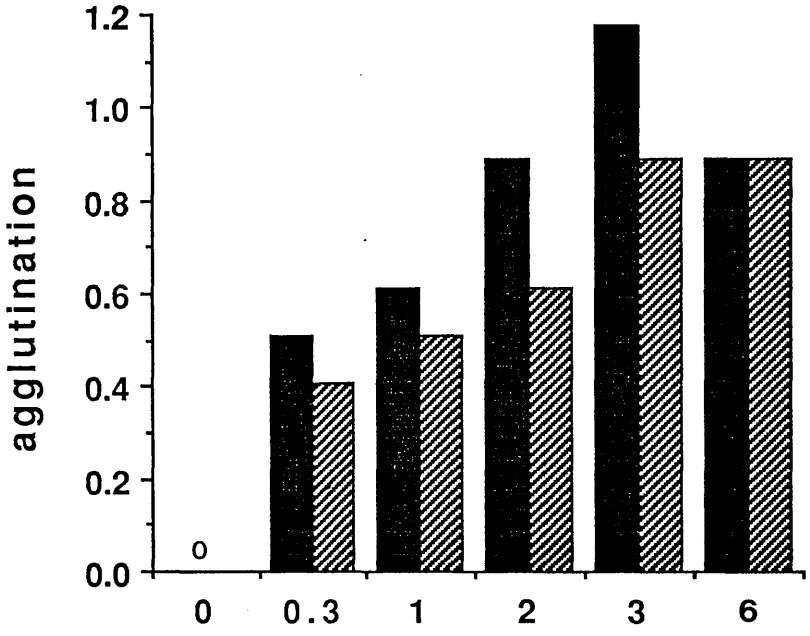
former are concerned with long term effects of parasite death and not with the events which occur during the first peak of a *T. brucei* infection. The experiments described in this chapter used a drug cure to remove parasites from the host bloodstream so that previously elicited antibody production can be observed.

Temporary drops in total IgM levels are also associated with parasitaemic remission and the marked range in IgM levels reported from normal (Tabel, 1979) to 24-fold increase (Kobayashi & Tizard, 1976) in *T. congolense* infections may be in part attributable to parasites mopping up specific IgM.

Table 5.1. Summary of results of agglutination and lysis assays.
NL = no lysis; NA = no agglutination; - = not tested

	GUTat7.2				GUTat7.13			
	Highest serum dilution giving				Highest serum dilution giving			
	>50% lysis		agglutination		>50% lysis		agglutination	
	cured	not cured	cured	not cured	cured	not cured	cured	not cured
0	NL	NL	NA	NA	NL	NL	NA	NA
8h	NL	NL	NA	NA	NL	NL	1/40	1/20
1d	-	-	-	-	NL	NL	1/80	1/40
2d	-	-	-	-	NL	NL	1/640	1/80
3d	NL	NL	1/2560	1/40	1/5	NL	1/5120	1/640
4d	NL	NL	1/1280	1/80	-	-	-	-
6d	NL	NL	1/1280	1/1280	1/5	1/5	1/640	1/640
7d	1/10	1/5	1/2560	1/1280	-	-	-	-

(a) Agglutination of GUTat 7.13 parasites



(b) Lysis of GUTat 7.13 parasites

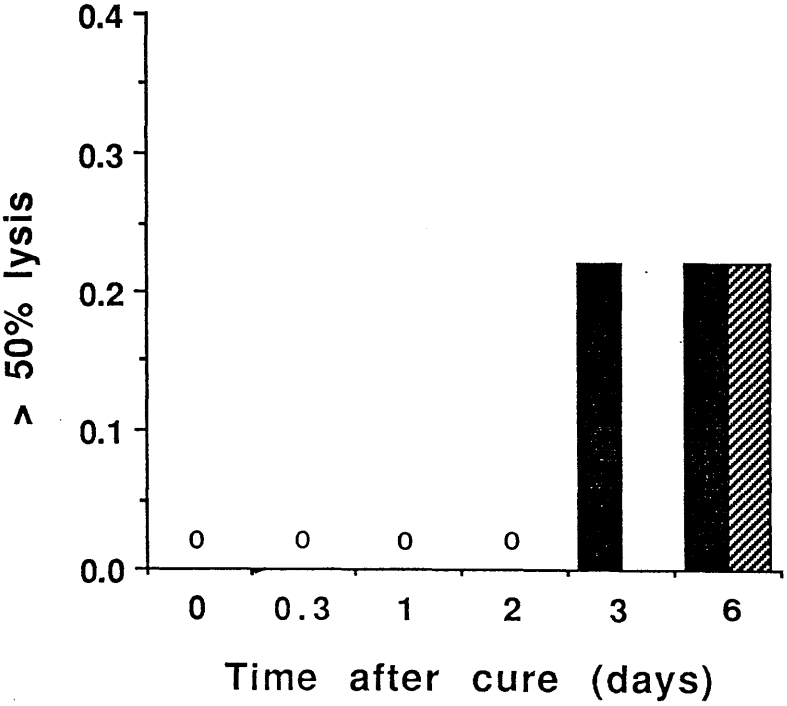


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Fig. 5.1. Agglutinating and lytic titres of serum samples collected from mice infected with GUTat 7.13.

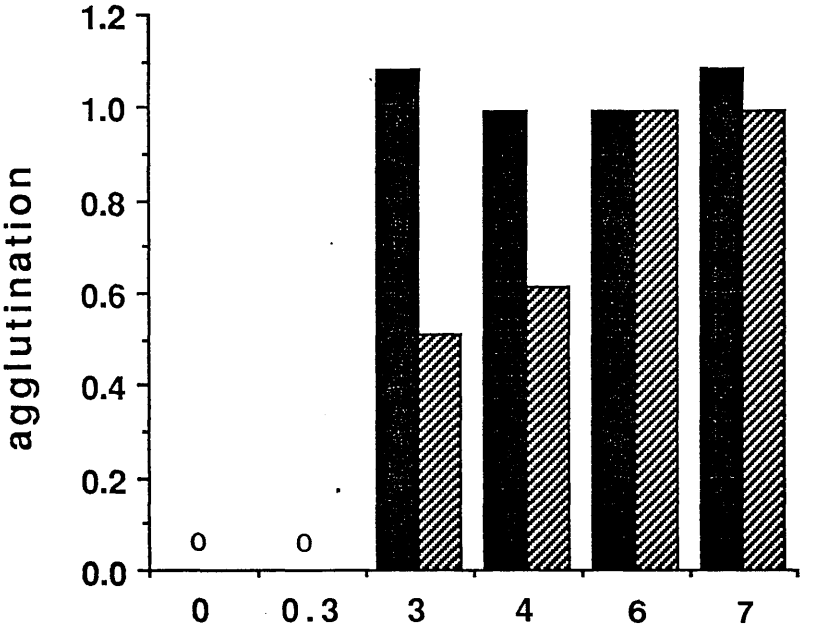
Sera from groups of BALB/c female mice infected with pleomorphic GUTat 7.13 were collected and pooled before and at time intervals after cure. The control mice were not cured, and the experimental mice were cured with SHAM-glycerol on day 3 of infection. (a) Log_2 of the reciprocal of the highest serum dilution giving 1+ agglutination of homologous trypanosomes at each time point. (b) Log_2 of the reciprocal of the highest serum dilution giving > 50% lysis of homologous trypanosomes at each time point.


Experimental group: cured on day 3 post-infection


Control group: infected but not cured

0 No agglutination/lysis

(a) Agglutination of GUTat 7.2 parasites



(b) Lysis of GUTat 7.2 parasites

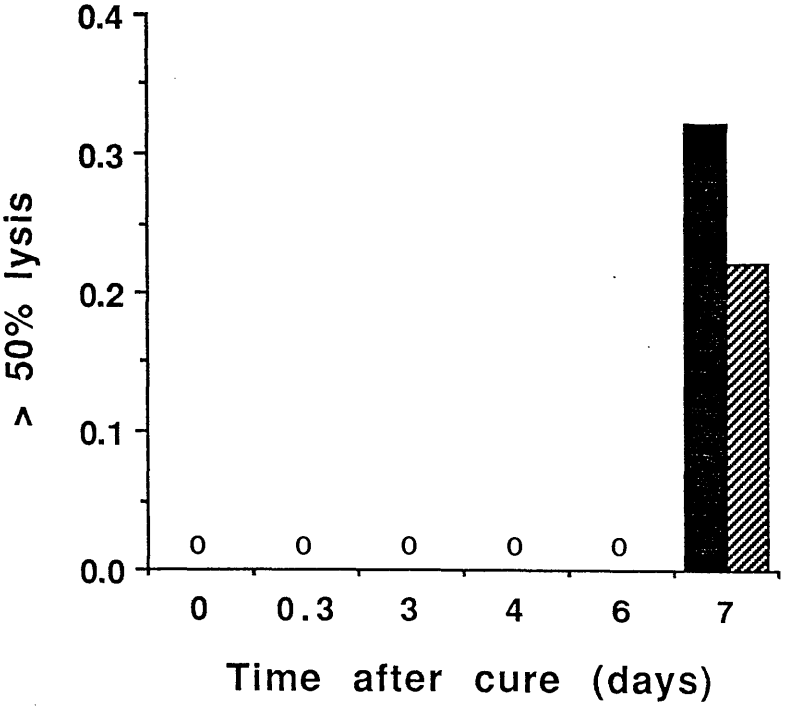


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Fig. 5.2. Agglutinating and lytic titres of serum samples collected from mice infected with GUTat 7.2.

Sera from groups of BALB/c female mice infected with pleomorphic GUTat 7.2 were collected and pooled before and at time intervals after cure. The control mice were not cured, and the experimental mice were cured with SHAM-glycerol on day 3 of infection. (a) Log_2 of the reciprocal of the highest serum dilution giving 1+ agglutination of homologous trypanosomes at each time point. (b) Log_2 of the reciprocal of the highest serum dilution giving > 50% lysis of homologous trypanosomes at each time point. Note that the vertical scale is expanded in this lower histogram.

Experimental group: cured on day 3 post-infection

Control group: infected but not cured

0 No agglutination/lysis

Chapter 6

GENERAL DISCUSSION

The experiments described in this thesis were conducted to elucidate (a) the mechanisms controlling pleomorphism in bloodstream African trypanosomes, and (b) the interactions of immune responses mounted by mammalian hosts with parasites of different morphological types.

The main features of bloodstream infections were initially documented early this century in experimental infections of rodents and monkeys with undefined isolates of "*T. brucei*" and "*T. gambiense*" (Robertson, 1912; Oehler, 1914a; Reichenow, 1921). The general characteristics of an infection are repeated cycles of rapid increase of parasitaemia followed by remission of infection. Within each cycle, slender forms predominate during the rising phase and stumpy forms predominate after the peak of parasitaemia.

All of the features of trypanosome infections described above were also observed in inbred mice infected with the cloned and antigenically homogeneous trypanosome line GUTat 7.13 (Chapter 2). This important observation indicated that such experimental infections are valid models of parasitaemic peaks in natural trypanosome infections. Therefore, this model system was used in all subsequent experiments described in this thesis.

Five models of differentiation were introduced in section 1.6.3. I return to these now to provide a framework for discussion of the mechanisms controlling pleomorphism. All of the models share a common assumption that the parasites in the infected hosts constitute a single systemic population. Although various subpopulations of trypanosomes are found in sites throughout the body, these non-bloodstream trypanosomes only account for about 10% of the total population. Also, there is a high rate of interchange of trypanosomes between at least one extravascular site and the bloodstream (Turner *et al.*, 1986).

Model (1). Differentiation occurs after a particular number of cycles of division.

There is evidence that this is the case in the free-living protozoa *Tetrahymena* and *Paramecium* (Elliott, 1973; Wichterman, 1986), but I am not aware of evidence for it in any parasitic protozoa. In particular it has been shown that for any trypanosome-mouse strain combination, parasitaemia maxima are similar, irrespective of the size of the parasite inoculum. This result is contrary to that predicted by this model (Seed & Sechelski, 1989).

Model (2). There is a definable probability that differentiation will occur after cell division.

This is a little explored area of the kinetics of trypanosome infections. Such a mechanism controls differentiation of pluripotent stem cells in mammals and gives the simplest explanation of the observation that

stumpy forms are produced at the greatest rate during the rising phase of the parasitaemia (Balber, 1972; section 2.2). This model alone cannot, however, explain the change from predominance of slender to predominance of stumpy forms observed at parasitaemic peak. A supplementary mechanism for removal of slender forms would be required.

Model (3). Division of trypanosomes is inhibited by cell-cell contact.

Contact inhibition is the phenomenon observed in cell culture whereby cells spread, move and divide until a monolayer is formed. The growth of normal cells is then inhibited, whereas neoplastic cells continue to grow. If such a culture of normal cells is damaged then the cells will again begin to divide and move into the damaged area. Such a mechanism is classically associated with the formation and healing of epithelia within multicellular organisms, but is not commonly associated with highly motile single-cell organisms.

There is direct evidence that contact inhibition does not occur in trypanosome infections: parasitaemia values estimate the number of parasites present per ml of blood, so that if contact inhibition limited growth then any given line of trypanosomes would always attain the same peak parasitaemia value. However, there are many documented examples of a trypanosome line reaching different peak parasitaemia levels in different mouse strains (see for example Black *et al.*, 1983; Seed & Sechelski, 1988b).

The fourth and fifth models are most easily dealt with together.

Model (4). Differentiation occurs/growth is inhibited when nutrient factors become limiting.

Model (5). There is a (non-nutrient) molecule which inhibits growth and/or promotes differentiation.

Black *et al.* (1985) proposed that differentiation is negatively regulated by the host. That is, trypanosomes multiply in the host bloodstream in response to a growth factor, and differentiate when the growth factor becomes depleted. My reservation about this model is that all the evidence is indirect (see section 1.6.3).

In Chapter 3 I attempted to test **directly** the role of depletion of a putative growth factor in trypanosome differentiation. If depletion of the putative growth factor triggers differentiation, then during the decline of the parasitaemia the growth factor would be present at too low a concentration to maintain cell division. Therefore a challenge population of slender form parasites superimposed onto a preexisting infection at the time of stumpy predominance would not be expected to grow. This was not found to be the case in experiments reported in Chapter 3: superimposed challenge infections grew to detectable levels in doubly-infected mice, and, moreover, grew at rates similar to those in singly-infected control mice.

Several interesting apparent anomalies were observed.

First, in one experiment (experiment 1) the growth rate of the superimposed challenge VAT was statistically significantly slower than in the controls: in the other experiments in which the growth rates were quantified rates were not significantly slower. Secondly, in one experiment (experiment 2) there was a delay of about 20h before growth of the challenge VAT could be detected in doubly-infected mice compared to controls. Although these two results could be construed as evidence for growth inhibition, they are not consistent observations and do not detract, therefore, from the general conclusion that depletion of a putative growth factor does not prevent trypanosome growth - the hypothesis of Black and colleagues states that no growth should occur **at all** when the preexisting parasitaemia is at its peak value.

The reasons for the slower growth rate and time lag phenomena are not clear. Possibly certain combinations of trypanosome lines interact with each other such that growth of one is suppressed, as has been previously postulated (Seed, 1978; Miller & Turner, 1981), although it has also been reported that growth rates in double infections do not differ from the growth rates of the same lines in single infections (Myler *et al.*, 1985). It is also possible that, even though growth factors to which trypanosomes may respond are not present in the host serum, nutrient supplies in the bloodstream immediately after a high peak in parasitaemia are limiting, at least in some hosts sometimes. Again different trypanosome lines might be

expected to have different threshold requirements for various nutrients, and therefore differ in their abilities to thrive immediately after a parasitaemic peak.

In the experiment where the challenge population was pleomorphic (experiment 4), although growth was not of the same magnitude as the controls, it did still occur. If growth had not occurred, the challenge VAT would not have been detectable. When the challenge VAT was detected, morphological analysis showed that the population was predominantly composed of stumpy forms. This high prevalence of stumpy forms was detected at t=168 h (i.e. 48 h) after inoculation of the challenge population. Since stumpy forms have a half life of 24-36h, slender forms capable of division must have been present during the intervening period of t=120 to t=168 h.

A fifth experiment (Table 3.4 in Chapter 3) also used a pleomorphic line as the challenge. In that experiment pleomorphic GUTat 7.2 was superimposed onto a preexisting infection of a pleomorphic line AnTat 1.8. The challenge population expanded and was detected by immunofluorescence. The expansion of the challenge population allowed the parasitaemia of the doubly-infected group to maintain a plateau value while the parasitaemia of the primary VAT control group quickly plunged below the level of detection. Thus, the growth pattern of the pleomorphic challenge VAT was very similar to those observed in experiments 1-3 in which the challenges were monomorphic lines of

trypanosomes. However, the total parasitaemia of the doubly-infected experimental group in experiment 5 maintained a plateau after inoculation of the pleomorphic challenge VAT, whereas in experiments 1-3 the parasitaemia level in the doubly-infected experimental groups rose after inoculation of the monomorphic challenge VATs.

Several aspects of this work would benefit from further experimentation. In particular it would be vital to repeat and extend the experiments using pleomorphic challenge populations because the findings reported in this thesis are not consistent. The limited availability of antigenically homogeneous pleomorphic lines would hinder such experiments, given that VAT and stock differences are necessary, and that specific antisera or monoclonal antibodies are required to detect the primary and challenge VATs in the mixed infections. More generally, possible differences between monomorphic and pleomorphic lines in their requirements for nutrients and growth factors needs further investigation.

In addition to the five models of trypanosome **differentiation** discussed above, I have considered a sixth factor that has a role in the change from predominance of slender forms to predominance of stumpy forms at the parasitaemic peak.

Model (6). This factor is VAT-specific antibody, and I conducted experiments to ascertain whether selective

clearance of slender form trypanosomes occurs. Preferential clearance of slender forms as the immune response develops, in conjunction with model (2) above would explain both the rapid rate of accumulation of stumpy forms during the rising phase of the parasitaemia and the change of morphology at the peak.

Preliminary evidence in support of selective clearance was available: lysis of slender form parasites in the presence of specific antibody and complement *in vitro* occurred more rapidly than lysis of stumpy forms (Barry & Vickerman, 1977). I chose to further this study *in vitro* because the experimental system could be controlled to ensure that the effects observed were due to the actions of specific antibody.

Two specific antibody-mediated anti-trypanosome effector mechanisms (complement-mediated lysis and phagocytosis of opsonised parasites) were investigated in Chapter 4. It was found that slender forms are more susceptible to lysis than stumpy forms expressing the same VAT, although no differences were observed in the attachment of slender and stumpy forms to phagocytic cells.

It is important to emphasise that the relative rates of lysis of slender and stumpy forms are not considered to constitute the cause of selective clearance *per se*. I have used these rates as a sensitive assay to index the relative resistance of trypanosomes of different morphological types

to antibody-mediated lysis. Differential lysis can also be observed by varying the concentration of antibody (C.M.R. Turner, unpublished results). Thus lysis is probably an important mechanism in the clearance of slender form trypanosomes that occurs once VAT-specific antibody responses have developed. The physical basis of the greater susceptibility of slender forms to lysis is not yet known.

To determine the importance of these findings within an overall framework that explains the morphological dynamics of an infection would require further experimentation *in vivo*: for example, inoculation of specific antiserum into mice in mid-infection when all morphological types are present followed by examination of drops of blood taken at time intervals after inoculation. The problem with such an experiment, however, is that dying trypanosome assume a variety of morphologies that cannot be reliably discriminated from stumpy forms.

Having considered selective clearance as a possible mechanism in pleomorphism, it was necessary to investigate an apparent shortcoming of model (6). From previous work (Black & Sendashonga, 1982; Sendashonga & Black, 1982), Black and colleagues had developed the theory that living trypanosomes cannot stimulate specific immune responses and that only VSG on fragments of dead parasites is immunogenic. This conclusion was based primarily on their inability to detect specific antibody in the serum of animals with monomorphic (slender form only) infections,

and on the lack of production of specific antibody from hybridomas made with spleen cells from animals with monomorphic infections. Black and colleagues considered, therefore, that death of non-dividing stumpy forms in the bloodstream is responsible for initiation of a VAT-specific immune response. This model is summarised in Fig. 6.1a. Obviously, if an antibody-mediated response is not generated against slender forms then selective clearance cannot occur.

Although Black and colleagues did not detect specific antibody in the serum of mice with monomorphic infections, directly contradictory findings have been published (Zahalsky & Weinberg, 1976). Also, it is difficult to imagine that a highly repetitive antigen such as VSG on dead parasites, but not on live ones, should stimulate the host immune system as was proposed by Black and colleagues. Instead, it is simpler to envisage that all bloodstream trypanosomes stimulate specific antibody responses but are able to bind large amounts of the antibody so that no free serum antibody titre is detectable early in infection. The rising phase of a trypanosome infection can be envisaged as a particularly effective antibody sink because of the rapid expansion of parasite numbers. To test this hypothesis (Chapter 5) the putative antibody sink was removed suddenly from the bloodstreams of infected mice by drug treatment, and serum samples were tested for the presence of specific antibody in agglutination and lysis assays. If live slender form parasites stimulated an immune response but acted as

an antibody sink, then newly-synthesised free serum antibody would be detected after drug cure. This was found to be the case: higher titres of specific antibody were detected in cured mice than in uncured mice at the same timepoint after infection. This showed that bloodstream trypanosomes did act as an antibody sink and that specific antibody was produced in significant amounts before a significant level of senescence occurred in the parasite populations. The greater sensitivity of the agglutination assay (compared with the lysis assay) in detection of serum antibody suggests that sufficient amounts of antibody for receptor-mediated adherence of opsonised trypanosomes to phagocytic cells can probably occur at lower specific antibody titres than lysis.

As a consequence of this finding, a simpler model of the induction of VAT-specific antibody responses in trypanosome infections can be proposed (Fig. 6.1b). According to this model, any living bloodstream trypanosome can stimulate an immune response in an immunocompetent host. VAT-specific antibodies bind to the VSG on the surfaces of the parasites and when sufficient titres are achieved opsonised parasites are ingested by phagocytic cells or lysed via the classical complement pathway. Free serum antibody titres are only detectable when there is antibody excess, and lack of specific antibody in the serum does not mean that there is no specific immune response.

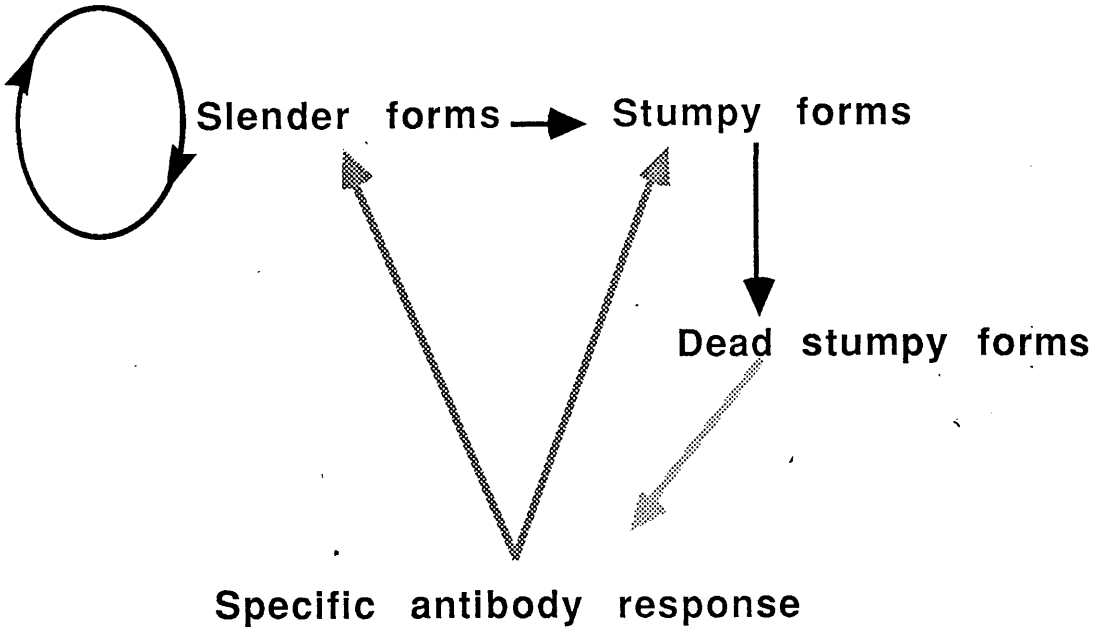
In conclusion, the regulation of pleomorphism in African

trypanosomes is a complex field in which it is not possible to determine the mechanisms by a few simple definitive experiments. Instead it is necessary to dissect the problem into smaller components and deal with each in turn. in light of the results it is possible to reject some models and construct the most likely model.

In this case, an explanation of pleomorphism has to accommodate the known characteristics of an infection. These are the accumulation of stumpy forms during the rising phase of the parasitaemia, and the change in morphological predominance at the peak. I conclude that differentiation to stumpy forms in African trypanosomes is a programmed event that has a defined probability at each cycle of slender form division (model 2), and that this, coupled with selective antibody-mediated clearance of slender forms (model 6), fulfils the requirements of the observed facts.

Whether these mechanisms exclusively control the dynamics of pleomorphism, however, is open to debate. The results in Chapter 3 do not provide strong support for the hypothesis that growth factors and/or nutrients may have a modifying role, but neither can they be used to formally exclude the possibility. The definitive experiments required to resolve this issue cannot be conducted until the critical growth factors and/or limiting nutrients have been identified, characterised and produced in quantities sufficient to permit manipulation of infections *in vivo*.

(a) Degenerating parasites stimulate immune response



Black, Hewett & Sendashonga. (1982) and Sendashonga & Black (1982)

(b) All parasites stimulate immune response

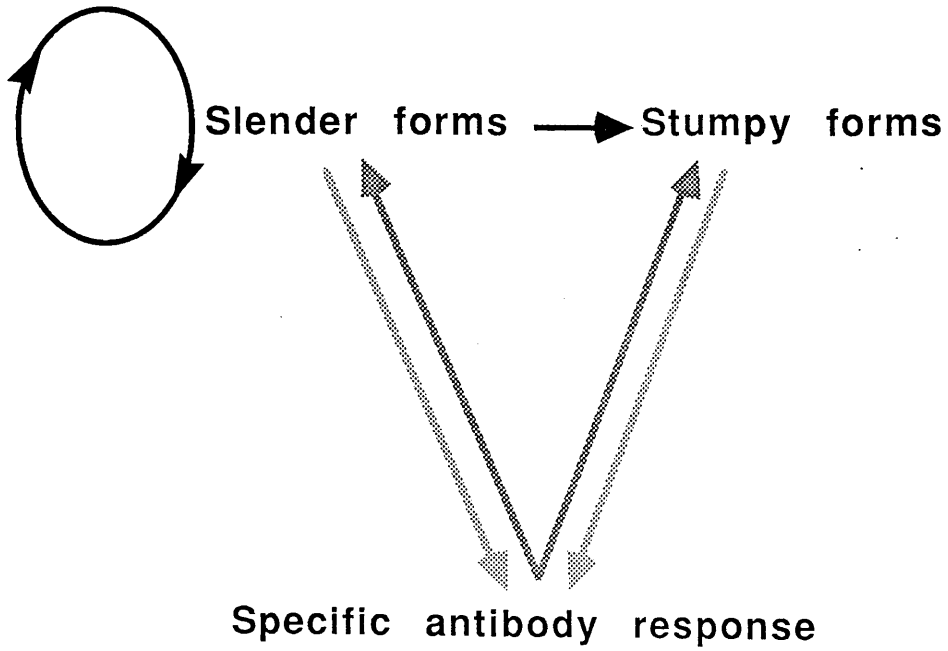


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Fig 6.1. (a) A model of induction of VSG-specific antibody responses derived from Black *et al.*, 1985. According to this model VSG on intact trypanosomes does not stimulate antibody production. Senescent trypanosomes, usually stumpy forms, are presented successfully to the B cells resulting in a VSG-specific antibody response. (b) Rational model in which trypanosome-bound VSG stimulates production of specific antibody. Detection of the antibody may be impaired in conditions of antigen excess such as occur during the rising phase of a parasitaemia.

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